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Eighteenth day of September 2000

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**The University of Melbourne
and
Commonwealth Scientific and Industrial
Research Organisation**

PROVISIONAL SPECIFICATION

Invention Title:

Vaccine antigens of Moraxella

The invention is described in the following statement:

Vaccine antigens of Moraxella

FIELD OF THE INVENTION

The present invention relates to antigens of *Moraxella*, in particular, *Moraxella bovis*, nucleic acid sequences encoding these antigens and
5 formulations for use in raising an immune response against *Moraxella*.

BACKGROUND OF THE INVENTION

Infectious bovine keratoconjunctivitis (IBK) is an economically important disease of cattle caused by the Gram-negative coccobacillus
10 *Moraxella bovis*. More commonly known as pinkeye, IBK is a highly contagious ocular infection which may range from mild conjunctivitis to severe ulceration, corneal perforation and blindness. Therapeutic and preventative measures have limited success in controlling IBK and a vaccine which will prevent the disease is required. A number of factors contribute to
15 the virulence of the organism, the two most important attributes so far identified are the expression of pili, and the ability to produce haemolysin. Seven different serogroups of *M. bovis* strains isolated in Australia, Great Britain and the USA have been characterised, based on pilus types (1). An efficacious pilus-based vaccine must contain a sufficient quantity of pili from
20 all seven serotypes to be fully protective, because of a lack of cross protection between serotypes (2, 3). Expression of all seven pilus serotypes at levels high enough to be useful for commercial vaccine preparation has not been achieved.

The ideal vaccine candidate to stimulate protection against *M. bovis*
25 would be a molecule that is highly-conserved among all strains of this species. Possible candidates are haemolysin, protease, lipase and/or phospholipase (4) enzymes produced by *M. bovis*. For example, a partially purified cell-free supernatant from one haemolytic strain of *M. bovis* has been shown to confer significant protection against heterologous, wild-type
30 challenge (5). The possibility that a haemolysin could be conserved across all seven serotypes of *M. bovis* makes it a potential vaccine candidate against IBK. However, researchers have so far been unable to either clone the gene encoding the haemolysin or purify the protein to homogeneity. Nevertheless, any or all of these molecules, alone or in combination, could prove useful for
35 the generation of an effective vaccine against IBK.

SUMMARY OF THE INVENTION

In a first aspect the present invention consists in a polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO. 1 from
5 amino acid 37 to 1114, or a sequence having at least 50% identity thereto, or a functional fragment thereof.

In a preferred embodiment the polypeptide has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 1.

10 In a further preferred embodiment of the present invention the polypeptide has protease activity.

In a second aspect the present invention consists in a nucleic acid molecule, the nucleic acid molecule encoding the polypeptide of the first aspect of the present invention.

15 In a preferred embodiment of this aspect of the present invention the nucleic acid molecule has a sequence as set out in SEQ. ID. NO. 2 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

In a preferred embodiment the nucleic acid molecule has a sequence of
20 at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 2.

In a third aspect the present invention consists in a composition for use in raising an immune response in an animal, the composition comprising the polypeptide of the first aspect of the present invention or the nucleic acid
25 sequence of the second aspect of the present invention and optionally a carrier and/or adjuvant.

In a fourth aspect the present invention consists in a polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO. 3 from amino acid 26 to 616, or a sequence having at least 50% identity thereto, or a
30 functional fragment thereof.

In a preferred embodiment the polypeptide has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 3 from amino acid 26 to 616.

In a further preferred embodiment of the present invention the
35 polypeptide has lipase activity.

In a fifth aspect the present invention consists in a nucleic acid molecule, the nucleic acid molecule encoding the polypeptide of the first aspect of the present invention.

5 In a preferred embodiment of this aspect of the present invention the nucleic acid molecule has a sequence as set out in SEQ. ID. NO. 4 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

10 In a preferred embodiment the nucleic acid molecule has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 4.

In a sixth aspect the present invention consists in a composition for use in raising an immune response in an animal, the composition comprising the polypeptide of the fourth aspect of the present invention or the nucleic acid sequence of the fifth aspect of the present invention and optionally a
15 carrier and/or adjuvant.

In a seventh aspect the present invention consists in a polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO. 5, or a sequence having at least 60% identity thereto, or a functional fragment thereof.

20 In a preferred embodiment the polypeptide has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 5.

In a further preferred embodiment of the present invention the polypeptide has haemolysin activity.

25 In an eighth aspect the present invention consists in a nucleic acid molecule, the nucleic acid molecule encoding the polypeptide of the first aspect of the present invention.

In a preferred embodiment of this aspect of the present invention the nucleic acid molecule has a sequence as set out in SEQ. ID. NO. 6 or a
30 sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

In a preferred embodiment the nucleic acid molecule has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 6.

35 In a ninth aspect the present invention consists in a composition for use in raising an immune response in an animal, the composition comprising

the polypeptide of the seventh aspect of the present invention or the nucleic acid sequence of the eighth aspect of the present invention and optionally a carrier and/or adjuvant.

5 The term "functional fragment" as used herein is intended to cover fragments of the polypeptide which retain at least 10% of the biological activity of the complete polypeptide. In particular this term is used to encompass fragments which show immunological cross-reactivity with the entire polypeptide, eg ligands which react with the fragment also react with the complete polypeptide.

10 In a tenth aspect the present invention consists in a composition for use in raising an immune response in an animal directed against *Moraxella*, the composition comprising at least one polypeptide selected from the group consisting of the polypeptides of the first, fourth and seventh aspects of the present invention and optionally including an adjuvant or carrier.

15 In a preferred embodiment the composition includes the polypeptide of the seventh aspect of the present invention and either one of, or preferably both, the polypeptides of the first and fourth aspects of the present invention.

In a preferred embodiment the *Moraxella* is *M. bovis* or *M. catarrhalis*, most preferably *M. bovis*.

20 In an eleventh aspect the present invention consists in an antibody raised against a polypeptide selected from the group consisting of the polypeptides of the first, fourth and seventh aspects.

25 As will be readily appreciated by the person skilled in this field the polypeptides and antibodies of the present invention and probes derived from the nucleotide sequences can be used as diagnostic reagents in determining *Moraxella*, in particular, *M. bovis* infection. For example, the polypeptides and antibodies can be used in ELISA based assays whilst the probes can be used in PCR based assays. The probes will be of a length to provide the required level of specificity and will typically be at of least 18
30 nucleotides in length.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements,
35 integers or steps.

DETAILED DESCRIPTION OF THE INVENTION

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following non-limiting Examples.

5

General Molecular Biology

Unless otherwise indicated, the recombinant DNA techniques utilized in the present invention are standard procedures, well known to those skilled
10 in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning:
A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes
15 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (Editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until
present) and are incorporated herein by reference.

20

Protein Variants

Amino acid sequence variants can be prepared by introducing appropriate nucleotide changes into DNA, or by *in vitro* synthesis of the
25 desired polypeptide. Such variants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final protein product possesses the desired characteristics. The amino acid changes also may alter
30 post-translational processes such as altering the membrane anchoring characteristics, altering the intra-cellular location by inserting, deleting or otherwise affecting the transmembrane sequences of the native protein, or modifying its susceptibility to proteolytic cleavage.

In designing amino acid sequence variants, the location of the
35 mutation site and the nature of the mutation will depend on characteristic(s) to be modified. The sites for mutation can be modified individually or in

series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of other ligands adjacent to the located site.

5 A useful method for identification of residues or regions for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (*Science* (1989) **244**: 1081-1085). Here, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged
10 amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants. Thus, while the site for introducing an amino acid sequence variation is
15 predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimise the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed variants are screened for the optimal combination of desired activity.

20 There are two principal variables in the construction of amino acid sequence variants; the location of the mutation site and the nature of the mutation. These may represent naturally occurring alleles or predetermined mutant forms made by mutating the DNA either to arrive at an allele or a variant not found in nature. In general, the location and nature of the
25 mutation chosen will depend upon the characteristic to be modified.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues.

Amino acid sequence insertions include amino and/or
30 carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Other insertional variants include the fusion of the N- or C-terminus of the proteins to an immunogenic polypeptide e.g. bacterial polypeptides such as betalactamase or an enzyme
35 encoded by the *E. coli trp* locus, or yeast protein, bovine serum albumin, and chemotactic polypeptides. C-terminal fusions with proteins having a

long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, are included.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the protein molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s). Other sites of interest are those in which particular residues obtained from various species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe norleucine	leu
Leu (L)	norleucine, ile; val; met; ala; phe	ile

Original Residue	Exemplary Substitutions	Preferred Substitutions
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile;	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Mutants, Variants and Homology - Proteins

5 Mutant polypeptides will possess one or more mutations which are deletions, insertions, or substitutions of amino acid residues. Mutants can be either naturally occurring (that is to say, purified or isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis on the encoding DNA). It is thus apparent that polypeptides of the invention can be either naturally occurring or recombinant (that is to say prepared using recombinant DNA techniques).

An allelic variant will be a variant that is naturally occurring within an individual organism.

Protein sequences are homologous if they are related by divergence from a common ancestor. Consequently, a species homologue of the protein will be the equivalent protein which occurs naturally in another species. Within any one species a homologue may exist as numerous allelic variants, and these will be considered homologues of the protein. Allelic variants and species homologues can be obtained by following standard techniques known to those skilled in the art. Preferred species homologues include those obtained from representatives of the same Phylum, more preferably the same Class and even more preferably the same Order.

A protein at least 50% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) *Ad. Appl Math.*, 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) *J. Mol. Biol.*, 48: 443-453), to that of the present invention are included in the invention, as are proteins at least 70% or 80% and more preferably at least 90% identical to the protein of the present invention. This will generally be over a region of at least 20, preferably at least 30, contiguous amino acids.

10 **Mutants, Variants and Homology - Nucleic Acids**

Mutant polynucleotides will possess one or more mutations which are deletions, insertions, or substitutions of nucleotide residues. Mutants can be either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis on the DNA). It is thus apparent that polynucleotides of the invention can be either naturally occurring or recombinant (that is to say prepared using recombinant DNA techniques).

An allelic variant will be a variant that is naturally occurring within an individual organism.

Nucleotide sequences are homologous if they are related by divergence from a common ancestor. Consequently, a species homologue of the polynucleotide will be the equivalent polynucleotide which occurs naturally in another species. Within any one species a homologue may exist as numerous allelic variants, and these will be considered homologues of the polynucleotide. Allelic variants and species homologues can be obtained by following standard techniques known to those skilled in the art. Preferred species homologues include those obtained from representatives of the same Phylum, more preferably the same Class and even more preferably the same Order.

A polynucleotide at least 70% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) *Ad. Appl Math.*, 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) *J. Mol. Biol.*, 48: 443-453), to that of the present invention are included in the invention, as are proteins at least 80% or 90% and more preferably at least 95% identical to the

polynucleotide of the present invention. This will generally be over a region of at least 60, preferably at least 90, contiguous nucleotide residues.

Antibody Production

5

Antibodies, either polyclonal or monoclonal, which are specific for a protein of the present invention can be produced by a person skilled in the art using standard techniques such as, but not limited to, those described by Harlow et al. *Antibodies: A Laboratory Manual*, Cold Springs Harbor Laboratory Press (1988), and D. Catty (editor), *Antibodies: A Practical Approach*, IRL Press (1988).

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of a protein. For the production of polyclonal antibodies, a number of host animals are acceptable for the generation of antibodies by immunization with one or more injections of a polypeptide preparation, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response in the host animal, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminium hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

A monoclonal antibody to an epitope of a protein may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256, 493-497), and the more recent human B-cell hybridoma technique (Kesber *et al.* 1983, *Immunology Today* 4:72) and EBV-hybridoma technique (Cole *et al.* 1985, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" by splicing the genes from an antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity may be used (Morrison *et al.* 1984, *Proc. Natl. Acad. Sci.*, 81:6851-6855; Neuberger *et al.* 1984 *Nature* 312:604-608; Takeda *et al.* 1985 *Nature*

31:452-454). Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce 4-specific single chain antibodies.

Recombinant human or humanized versions of monoclonal antibodies
 5 are a preferred embodiment for human therapeutic applications. Humanized antibodies may be prepared according to procedures in the literature (e.g. Jones *et al.* 1986, Nature 321:522-25; Reichman *et al.* 1988, Nature 332:323-27; Verhoeyen *et al.* 1988, Science 239:1534-36). The recently described "gene conversion mutagenesis" strategy for the production of
 10 humanized monoclonal antibody may also be employed in the production of humanized antibodies (Carter *et al.* 1992 Proc. Natl. Acad. Sci. U.S.A. 89:4285-89). Alternatively, techniques for generating the recombinant phage library of random combinations of heavy and light regions may be used to
 15 prepare recombinant antibodies (e.g. Huse *et al.* 1989 Science 246:1275-81).

Antibody fragments which contain the idiotype of the molecule such
 15 as $F(ab^1)$ and $F(ab^2)$ may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab)$ E2 fragment which can be produced by pepsin digestion of the intact antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the
 20 $F(ab')_2$ fragment, and the two Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Alternatively, Fab expression libraries may be constructed (Huse *et al.* 1989, Science 246:1275-1281) to allow rapid and easy cloning of a monoclonal Fab fragment with the desired specificity to a protein.

25

Adjuvants and Carriers

Pharmaceutically acceptable carriers or diluents include those used in compositions suitable for oral, rectal, nasal, topical (including buccal and
 30 sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. They are non-toxic to recipients at the dosages and concentrations employed. Representative examples of pharmaceutically acceptable carriers or diluents include, but are not limited to water, isotonic solutions which are preferably
 35 buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline) and can also contain one or more of, mannitol, lactose,

trehalose, dextrose, glycerol, ethanol or polypeptides (such as human serum albumin). The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy.

5 As mentioned above the composition may include an adjuvant. As will be understood an "adjuvant" means a composition comprised of one or more substances that enhances the immunogenicity and efficacy of a vaccine composition. Non-limiting examples of suitable adjuvants include squalane and squalene (or other oils of animal origin); block copolymers; detergents
 10 such as Tween®-80; Quil® A, mineral oils such as Drakeol or Marcol, vegetable oils such as peanut oil; *Corynebacterium*-derived adjuvants such as *Corynebacterium parvum*; *Propionibacterium*-derived adjuvants such as *Propionibacterium acne*; *Mycobacterium bovis* (Bacille Calmette and Guérin or BCG); interleukins such as interleukin 2 and interleukin 12; monokines
 15 such as interleukin 1; tumour necrosis factor; interferons such as gamma interferon; combinations such as saponin-aluminium hydroxide or Quil-A aluminium hydroxide; liposomes; ISCOM adjuvant; mycobacterial cell wall extract; synthetic glycopeptides such as murarmyl dipeptides or other derivatives; Avridine; Lipid A derivatives; dextran sulfate; DEAE-Dextran or
 20 with aluminium phosphate; carboxypolymethylene such as Carbopol' EMA; acrylic copolymer emulsions such as Neocryl A640 (e.g. U.S. Pat. No. 5,047,238); vaccinia or animal poxvirus proteins; sub-viral particle adjuvants such as cholera toxin, or mixtures thereof.

25 **Gene/DNA Isolation**

The DNA encoding a protein may be obtained from any cDNA library prepared from tissue believed to express the gene mRNA and to express it at a detectable level. DNA can also be obtained from a genomic library.

30 Libraries are screened with probes or analytical tools designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal or polyclonal antibodies that recognize and specifically bind the protein; oligonucleotides of about 20-80 bases in length that encode known or suspected portions of
 35 cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments thereof that encode the same or a

hybridizing gene. Appropriate probes for screening genomic DNA libraries include, but are not limited to, oligonucleotides; cDNAs or fragments thereof that encode the same or hybridizing DNA including expressed sequence tags and the like; and/or homologous genomic DNAs or fragments thereof.

- 5 Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook *et al.*

An alternative means to isolate a gene encoding the protein of interest is to use polymerase chain reaction (PCR) methodology as described in
10 section 14 of Sambrook *et al.* This method requires the use of oligonucleotide probes that will hybridize to the gene.

The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false-positives are minimized. The actual nucleotide sequence(s) is usually based on conserved
15 or highly homologous nucleotide sequences or regions of the gene. The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage in that species is known. The oligonucleotide must be labelled such that it can be detected
20 upon hybridization to DNA in the library being screened. The preferred method of labelling is to use (α -³²P)- dATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labelling.

25 DNA encompassing all the protein coding sequence is obtained by screening selected cDNA or genomic libraries, and if necessary, using conventional primer extension procedures as described in section 7.79 of Sambrook *et al.*, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

30 Another alternative method for obtaining the gene of interest is to chemically synthesize it using one of the methods described in Fingels *et al.* (*Agnew Chem. Int. Ed. Engl.* 28: 716-734, 1989). These methods include triester, phosphite, phosphoramidite and H-Phosphonate methods, PCR and other autoprimer methods, and oligonucleotide syntheses on solid supports.
35 These methods may be used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding

strand is available, or alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue.

5 **Substantially Purified**

By "substantially purified" we mean a polypeptide that has been separated from lipids, nucleic acids, other polypeptides, and other contaminating molecules.

10 The polynucleotide sequence of the present invention may hybridise to the respective sequence set out SEQ. ID. NOS. 2, 4, or 6 under high stringency. As used herein, stringent conditions are those that (i) employ low ionic strength and high temperature for washing after hybridization, for example, 0.1 x SSC and 0.1% (w/v) SDS at 50°C; (ii) employ during
15 hybridization conditions such that the hybridization temperature is 25°C lower than the duplex melting temperature of the hybridizing polynucleotides, for example 1.5 x SSPE, 10% (w/v) polyethylene glycol 6000, 7% (w/v) SDS, 0.25 mg/ml fragmented herring sperm DNA at 65°C; or (iii) for example, 0.5M sodium phosphate, pH 7.2, 5mM EDTA, 7% (w/v) SDS
20 and 0.5% (w/v) BLOTTO at 70°C; or (iv) employ during hybridization a denaturing agent such as formamide; for example, 50% (v/v) formamide with 5 x SSC, 50mM sodium phosphate (pH 6.5) and 5 x Denhardt's solution (32) at 42°C; or (v) employ, for example, 50% (v/v) formamide, 5 x SSC, 50mM sodium phosphate (pH 6.8), 0.1% (w/v) sodium pyrophosphate, 5 x
25 Denhardt's solution, sonicated salmon sperm DNA (50µg/ml) and 10% dextran sulphate at 42°C.

EXAMPLE 1

30 This example describes the cloning and characterisation of a protease from *Moraxella bovis*.

Bacteria and construction of a genomic library

35 *Moraxella bovis* strain Dalton 2d was a field isolate collected from a bovine eye and characterised by CSIRO Animal Health, Parkville, Australia (6). *Escherichia coli* strain DH5α has been previously described (7, 8).

All enzymes were purchased from Promega (Madison, WI, USA) except where otherwise noted.

General cloning and DNA techniques were as described (9) unless otherwise noted.

- 5 A genomic library was constructed by carrying out partial *Sau*3A digests on genomic DNA extracted from *M. bovis* strain Dalton 2d using a CTAB method which is outlined below. This DNA was size fractionated using a NaCl gradient (10) and ligated with the cosmid cloning vector pHC79 (11) which had been previously digested with *Bam*HI. This DNA was
10 packaged into lambda bacteriophage heads using the Packagene Lambda DNA packaging system (Promega, Madison, WI, USA) and this was used to transduce the *E. coli* strain DH5 α . The library was stored in 96 well trays (50% glycerol / luria broth / ampicillin (50 μ g/ml)) at -70°C.

15 **CTAB genomic DNA extraction from *M. bovis***

- A 5ml brain heart infusion (BHI) (Oxoid Ltd., Basingstoke, Hampshire, U.K.) broth was inoculated with a colony of Dalton 2d taken from a fresh overnight culture on horse blood agar and incubated with shaking at 37°C for 6 hours. This culture was used to inoculate 50ml of BHI broth which was
20 grown with shaking at 37°C overnight. 40ml of the culture was transferred to an SS34 tube and the cells pelleted at 3000 \times g for 10 minutes. Following resuspension of the pellet in 9.5ml of 25% sucrose in TE buffer (10mM Tris, 1mM EDTA (pH8)), 500 μ l of 10% SDS, 50 μ l of 20mg/ml proteinase K and 20 μ l of 10mg/ml RnaseA were added and this mixture incubated in an orbital
25 shaker for 1 hour at 37°C. To this mixture, 1.8ml of 5M NaCl and 1.5ml of a CTAB (N-Cetyl-N,N,N-trimethyl-ammonium bromide) / NaCl solution was added and incubation continued for 20 minutes at 65°C. The DNA was extracted using phenol/chloroform and precipitated with 0.6 volumes of isopropanol. The resulting DNA was washed in 70% ethanol, dried and
30 resuspended in 2ml of TE buffer.

Screening of genomic library for enzyme activity

- The genomic library was cultured on skim milk agar to screen for the presence of a clone displaying protease activity (double strength Columbia
35 agar base (Oxoid Ltd., Basingstoke, Hampshire, U.K.) / 10% skim milk) for 24 hours at 37°C followed by refrigeration at 4°C for one to two days.

A single clone from the genomic library was detected as having activity against skim milk agar (Figure 1). DNA analysis confirmed that the clone contained a fragment of *M. bovis* Dalton 2d genomic DNA approximately 40 kilobases in size. The construct was designated pJF1.

5

Nucleotide sequence of the *M. bovis* protease clone pJF1

Plasmid and cosmid DNA for automated sequencing was extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) and the Qiagen Plasmid Midi Kit (Qiagen Pty. Ltd., Clifton Hill, Vic, Australia), respectively.

The nucleotide sequence of the insert DNA was determined using the process of "primer walking" (12). This was achieved using synthetic oligonucleotides (Bresatec/ Geneworks, Thebarton, SA, Australia) and the dye terminator cycle sequencing ready reaction (Perkin Elmer Corporation, Norwalk, CT, USA). The resulting sequence was analysed on an Applied Biosystems 373A DNA sequencer.

Automated sequencing revealed an open reading frame of 3345bp capable of encoding a protein of 1115 amino acids. The sequence is written in the 5' to 3' direction and is shown in Figure 2 together with the corresponding amino acid sequence which is predicted to encode a protein with a molecular weight of 120kDa. The amino acid sequence is shown in SEQ. ID. NO. 1 and the DNA sequence is shown in SEQ. ID. NO. 2.

The putative start codon for the mature protease protein was identified by the presence of a possible ribosome binding site upstream. This RBS was identified by its similarity to the consensus sequence for the *E. coli* RBS and that previously identified for the *M. bovis* pilin genes (AGGAG) (27)

Due to the secreted nature of the protease, it was assumed that it would contain in its N-terminal sequence a signal peptide which would be used in the secretion of the protein. This analysis was carried out using a prediction program (SignalP) available through the Expasy website (<http://www.expasy.ch/tools/>), which allows for the identification of prokaryotic signal peptides and predicts possible cleavage sites. This analysis only identified a signal peptide using the start codon indicated in the accompanying protein/DNA sequence.

35

Sequence comparisons

Comparisons of the deduced amino acid sequence with those in the database were carried out using the BlastX and BlastP programs (13) which are available at <http://www.ncbi.nlm.nih.gov>.

- 5 At the amino acid level, the protease cloned from Dalton 2d displayed the following similarity and identity to the proteins listed.

Organism	Protein	Similarity	Identity
<i>Serratia marcescens</i>	ssp-h2 - serine protease autotransporter	39%	23%
<i>Serratia marcescens</i>	ssp-h1 - serine protease autotransporter	37%	22%
<i>Pseudomonas flourescens</i>	serine protease homologue	34%	20%
<i>Pseudomonas tolaasii</i>	serine protease	35%	21%

- 10 More generally the 5' domain of the *M. bovis* protease displays homology to a family of subtilisins (serine proteases) while the 3' region resembles a number of outer membrane proteins.

The *M. bovis* sequence was found to contain a highly proline rich region which distinguished it from all other proteins to which it was closely related.

15

Protease type encoded by pJF1

In order to identify the type of protease activity encoded by pJF1, a range of specific protease inhibitors were examined for their effect on the expression of the *M. bovis* protease.

- 20 The method of Bourgeau *et al.*, (1992) (14) was used to determine inhibitor activity with the following modifications. 100µl of cell free supernatant from a fresh overnight broth culture was mixed with 650µl of 100mM Tris (pH 7.2) and a suitable volume of inhibitor PMSF (phenylmethylsulfonyl fluoride) 5mM; EDTA 5mM; leupeptin 100µg/ml; 25 pepstatin 50µg/ml]. Distilled water was used to make the volume up to 1ml. The mixture was incubated at 37°C for 30 minutes and 10mg of azocoll (Calbiochem, Alexandria, NSW, Australia) was then added. The suspensions were incubated at 37°C for 16 hours and the optical density read at 520nm.

In this way it was confirmed that the activity attributable to the protease encoded by pJF1 was that of a serine protease since PMSF (a serine protease inhibitor) reduced the protease activity of both Dalton 2d and pJF1 to zero.

5

Conservation of protease in *M. bovis*

Southern hybridisation using an internal fragment of the protease coding region as a probe was carried out to investigate whether the protease was present in strains representing the known *M. bovis* pili serotypes.

10

Genomic DNA extracted from the representative strains of *M. bovis* (15) was digested with *Xba*I and *Eco*RI and separated using agarose gel electrophoresis. The DNA was transferred to a Hybond N+ filter (Amersham, Little Chalfont, Buckinghamshire, U.K.) using the method described (9). The probe used in the southern hybridisation was a PCR amplified fragment which was internal to the protease coding region. This fragment was labelled with $\alpha^{32}\text{P}$ -dATP using the Megaprime labelling system (Amersham, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturers instructions. High stringency conditions were used (hybridisation temperature 68°C; 2 washes at room temperature in $2 \times \text{SSC} / 0.1\% \text{SDS}$; 1 wash at 68°C in $0.1 \times \text{SSC} / 0.1\% \text{SDS}$) and the resulting filters were exposed to autoradiographic film (Kodak, Rochester, New York, USA) for 5 to 24 hours before developing.

15

20

Figure 3 illustrates that the protease gene cloned in pJF1 is present in all strains of *M. bovis* examined.

25

EXAMPLE 2

This example describes the cloning and characterisation of a lipase from *Moraxella bovis*.

30

Bacteria and construction of a plasmid library

Moraxella bovis strain Dalton 2d was a field isolate collected from a bovine eye and characterised by CSIRO Animal Health, Parkville, Australia (6). *Escherichia coli* strain MC1061 has been previously described (16).

All enzymes were purchased from Promega (Madison, WI, USA) except where otherwise noted. General cloning and DNA techniques were as described (9) unless otherwise noted.

A plasmid library was constructed in the cloning vector pBR322 (17).
5 This was done by partially digesting genomic DNA extracted from Dalton 2d (using the CTAB method described in Example 1) with *Sau*3A under conditions that maximised the amount of DNA in the range of 1 to 2kb. This DNA was ligated with pBR322 which had been previously digested with *Bam*HI. The ligated DNA was electroporated (2.5kV, 200 Ω and 200 μ F, for a
10 theoretical time constant of 4.7) into electrocompetent *E. coli* MC1061 cells.

Screening of plasmid library for lipase expression

Following electroporation of the ligated DNA into MC1061 cells, recombinant clones displaying lipase activity were detected by culturing the
15 library for 24 hours at 37°C on media containing Tween 80 [10ml Tween 80 (Sigma, St Louis, MO, USA), 5g NaCl, 3g agar No.1 (Oxoid Ltd., Basingstoke, Hampshire, U.K.), 10g peptone, 0.1g CaCl₂·H₂O / litre].

Twenty eight out of 24,000 clones screened were found to be displaying lipase activity. DNA analysis confirmed that all of these clones
20 contained one 5.4kb fragment of DNA in common. One clone was chosen to continue work with and this was designated pMB1 (Figure 4).

In some experiments (below), a photometric assay of extracellular lipase activity was performed with p-nitrophenylpalmitate as the substrate (18, 19). Strains of *E. coli* and/or *M. bovis* were grown at 37°C for the
25 required time points. Cell free culture supernatant (100 μ l) was mixed with 2.4ml of enzyme buffer (19) to assay secreted lipase activity. After 30 minutes incubation at 37°C, the optical density at 410nm was determined. One enzyme unit was defined as the amount of enzyme that releases 1 nmol of p-nitrophenyl from p-nitrophenylpalmitate ml⁻¹ min⁻¹. Under the
30 conditions described by Stuer *et al.*, (18), an optical density at 410nm of 0.041 is equivalent to 1 enzyme unit.

Nucleotide sequence of the *M. bovis* lipase clone pMB1

Plasmid pMB1 was subjected to automated DNA sequencing using the
35 methodology described in Example 1.

This analysis revealed an open reading frame of 1851bp capable of encoding 617 amino acids. The sequence is written in the 5' to 3' direction and is shown in Figure 5 together with the corresponding amino acid sequence that is predicted to encode a protein with a molecular weight of 65.8kDa. The amino acid sequence is shown in SEQ. ID. NO. 3 and the DNA sequence is shown in SEQ. ID. NO. 4.

The techniques set out above in respect of the protease were used to identify the potential start codon for the lipase protein.

10 Sequence comparisons

Sequence comparisons were made using the methodology described in Example 1.

At the amino acid level, the lipase cloned from *M. bovis* Dalton 2d was shown to display the following similarity and identity to the proteins listed.

15

Organism	Protein	Similarity	Identity
<i>Xenorhabdus luminescens</i>	triacylglycerol lipase	36%	24%
<i>Pseudomonas putida</i>	hypothetical protein	36%	24%
<i>Salmonella typhimurium</i>	outer membrane esterase	35%	23%
<i>Pseudomonas aeruginosa</i>	lipase / esterase	36%	23%

The *M. bovis* lipase was identified as being a possible new member of the GDSE family (20) of lipolytic enzymes.

20 N-terminal sequencing carried out on the lipase mature protein

The required strains of *E. coli* were cultured overnight with shaking at 37°C in 500mls of luria broth. The cells were pelleted at 5,000 rpm for 15 mins and the supernatant filtered through a 0.45µm filter. Solid ammonium sulfate was added to the supernatant to 60% saturation (180g / 500ml), and dissolved at 4°C with stirring for 30 minutes. This mixture was left at 4°C overnight and the precipitated proteins pelleted at 7,000 rpm for 30 mins. The proteins were resuspended in 3ml of double distilled water and the solubilised proteins dialysed against double distilled water overnight to

remove any salt. The resulting mixture was filtered through a 0.45µm filter and stored at -20°C.

Following separation of the proteins by SDS-PAGE, the proteins were transferred to PVDF membrane and excised. The protein was subjected to automated (Edman degradation) sequence analysis (28) with vapour phase delivery of critical reagents (29) in an automated sequenator (model 470A; Applied Biosystems) (Applied Biosystems Division, Foster City, CA, USA) in conjunction with a PTH amino acid separation system (model 120A PTH analyzer; Applied Biosystems).

Using this technique 17 amino acids with two gaps were identified

K E F S Q V I I F G D S L ? D ? G

which corresponds exactly with amino acids 26 through to 42 shown on the accompanying sequence. This result also indicated that the protein most likely includes an amino terminal signal peptide which is involved in the secretion of the protein. This amino terminal corresponds to amino acids 1 through to 25 in the accompanying sequence.

Raising antibodies to the lipase in rabbits

Antibody to the recombinant lipase was raised in rabbits by injecting ammonium sulfate precipitated supernatant from *E. coli* MC1061/pMB4. Prior to vaccination, the lipase preparation was inactivated by heating to 90°C for 90min. 30µg of this protein was injected at 2 weekly intervals for 4 weeks. The primary inoculum was emulsified with Freund's complete adjuvant and subsequent vaccinations with Freund's incomplete adjuvant.

Heat stability of *M. bovis* lipase

The recombinant lipase cloned from *M. bovis* Dalton 2d was found to be very heat stable since it required heating at 90°C for 105 minutes for the activity to be reduced by 97%. Figure 6 illustrates this phenomenon with enzyme activity expressed as "lipase enzyme units" as determined in the extracellular lipase assay.

Relative expression levels of native versus recombinant lipase

An experiment was performed to plot growth rate with lipase production and to compare production of the recombinant lipase from MC1061/pMB1 with that of the native form of the lipase from *M. bovis* Dalton

2d. Figure 7(a) illustrates that the two strains grow at approximately the same rate but they do not reach the same cell density, with Dalton 2d substantially lower after 9 hours than MC1061/pMB1. Lipase expression levels were greatest from the pMB1 construct in *E. coli* compared to native lipase expression from *M. bovis* Dalton 2d.

This result was further substantiated when proteins from cell-free supernatants of either the *E. coli* clone or *M. bovis* Dalton 2d were ammonium sulfate precipitated and analysed by SDS-PAGE and western blot using antisera to the recombinant heat-deactivated lipase.

Ammonium sulfate precipitated supernatants were prepared from overnight cultures of *E. coli* or *M. bovis* that had been shaken at 37°C in either 500mls of Luria broth or brain heart infusion broth, respectively. Cells were pelleted at 5000 × g for 15 minutes and the supernatant filtered through a 0.45µm filter. Solid ammonium sulfate was added to the supernatant to 60% saturation (180g / 500ml) and dissolved at 4°C with stirring for 30 minutes. This mixture was left at 4°C overnight and the precipitated protein pelleted at 7000 × g for 30 minutes. Proteins were resuspended in 3ml of double distilled water and the solubilised proteins dialysed against double distilled water overnight to remove any salt. The resulting mixture was filtered through a 0.45µm filter and stored at -20°C.

Protein samples (100µl) were prepared for SDS-PAGE by resuspension in 100µl of 2x sample buffer (5ml 0.5M Tris (pH6.8), 8ml 10% SDS, 4ml glycerol, 0.8ml β-mercaptoethanol, 1ml double distilled H₂O, bromophenol blue) and heating to 100°C for 5 minutes. The proteins were separated on a 12.5% polyacrylamide gel using the buffer system of Laemmli (21).

Western blots were carried out according to the method of Towbin *et al.*, (22) and following separation of proteins by SDS-PAGE and transfer to nitrocellulose using the Bio-Rad minicell (Bio-Rad, Hercules, CA, USA) transfer system. Filters were immunoblotted with the recombinant lipase antiserum (at a concentration of 1/100) which had been adsorbed against MC1061 cells. The antiserum was raised against ammonium sulfate precipitated recombinant lipase which had been heat deactivated (1 hour 45 minutes at 90°C) and used to inoculate rabbits (three doses of 50µg each) at 4 week intervals. Blood samples were collected from the marginal ear vein prior to immunisation and at each vaccination time point.

Figure 7(b) shows a prominent band present in the recombinant lipase positive construct MC1061/pMB1 that is detectable in relatively minor amounts in *M. bovis* Dalton 2d preparation. The protein detected with the antisera was approximately the same size as that of the predicted molecular weight for the *M. bovis* lipase (65.8kDa).

Lipase type encoded by pMB1

Thin layer chromatography (TLC) was used to determine whether the lipase of *M. bovis* Dalton 2d displayed phospholipase activity. Characterisation of phospholipase type essentially followed a previously described method (23) except that the results of separation on Silica Gel 60 plates were visualised by developing with a 10% ethanolic solution of molybdophosphoric acid at 100°C. All reagents used were purchased from Sigma (Sigma, St Louis, MO, USA).

TLC determined that the *M. bovis* lipase displayed the same enzyme specificity as that of a commercially-available phospholipase B when lysophosphatidylcholine and phosphatidylcholine were used as substrates (data not shown).

Conservation of lipase among *M. bovis*

A southern blot using an internal fragment of the Dalton 2d lipase coding region was used to investigate whether the lipase gene was present in strains of *M. bovis* representing the known pilus serotypes.

Genomic DNA extracted from the strains of *M. bovis* representing each of the known pilus serotypes (15) was digested with *Hind*III and separated using agarose gel electrophoresis. The DNA was transferred to a Hybond N+ filter (Amersham, Little Chalfont, Buckinghamshire, U.K.) using a previously described method (9). The probe used in the southern hybridisation was a *Hind*III fragment that contained sequence internal to the lipase coding region. This fragment was labelled with $\alpha^{32}\text{P}$ -dATP using the Megaprime labelling system (Amersham, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturers instructions. High stringency conditions were used (hybridisation temperature 68°C; 2 washes at room temperature in $2 \times \text{SSC} / 0.1\% \text{SDS}$; 1 wash at 68°C in $0.1 \times \text{SSC} / 0.1\% \text{SDS}$) and the resulting filters were exposed to autoradiographic film (Kodak, Rochester, New York, USA) for 5 to 24 hours before developing.

Figure 8 illustrates that the lipase gene is present in all strains of *M. bovis* examined.

To confirm whether or not the lipase gene was expressed in each of the serotype representative strains, antisera raised against recombinant heat deactivated lipase was used in a western blot analysis of whole cell preparations. Figure 9 shows that the lipase was indeed being expressed by all of these *M. bovis* strains.

EXAMPLE 3

Bacteria and construction of a haemolysin clone

Moraxella bovis strain Dalton 2d was a field isolate collected from a bovine eye and characterised by CSIRO Animal Health, Parkville, Australia (6).

All of the *M. bovis* strains representative of the known pilus serotypes express a haemolytic activity that is detected on horse blood agar (Figure 10).

Escherichia coli strain degP4::Tn5 has a leaky outer membrane and is defective in proteolysis and has been previously described (24).

All enzymes were purchased from Promega (Madison, WI, USA) except where otherwise noted.

General cloning and DNA techniques were as described (9) unless otherwise noted.

A *phoA* fusion technique that allows for the identification of exported proteins (25) was utilised with some modifications. Genomic DNA from *M. bovis* Dalton 2d (prepared using the CTAB method described in Example 1) was partially digested with *Sau3A*. Restricted DNA was ligated with a series of vectors that allow fusions with an alkaline phosphatase gene in three different reading frames. The ligated DNA was electroporated into *E. coli* degP4::Tn5 and the resulting clones screened on Luria agar containing ampicillin (50µg/ml) and X-P (200µg/ml) (5-bromo-3-chloro-indolyl phosphate). Selection of clones relies on the observation that if the fragment is cloned in frame and contains an export sequence the resulting colony will be blue in colour. The leaky *E. coli* strain allows the outer membrane-bound proteins and secreted proteins (both fused with *phoA*) to be distinguished from non-secreted fusion proteins.

Sequencing of the *M. bovis* haemolysin determinant

Clones selected for the presence of a secreted or outer membrane protein gene sequence were subjected to automated DNA sequencing using the methods described in Example 1. One of these clones, pMbh1, was found to contain 200bp of DNA which displayed high homology to the A subunit of other RTX toxins. Inverse PCR and degenerate oligonucleotides were utilised to obtain the sequence of the entire A subunit. The open reading frame of 2784bp was capable of encoding 928 amino acids. The sequence is written in the 5' to 3' direction and is shown in Figure 11 together with the corresponding amino acid sequence that is predicted to encode a protein with a molecular weight of 98.8kDa. The amino acid sequence is shown in SEQ. ID. NO. 5 and the DNA sequence is shown in SEQ. ID. NO. 6.

The putative start codon was identified using the RBS technique outlined above. A signal peptide analysis was not carried out as the A subunit is not secreted on its own. However as the protein sequence of these proteins (RTX) is quite highly conserved, on amino acid homologies alone this start codon was the one of choice.

Sequence homology

At the amino acid level the *M. bovis* Dalton 2d haemolysin gene product shows striking similarity to the A subunit of the of several RTX and other haemolysins as shown in the following table.

Organism	Protein	Similarity	Identity
<i>Pasteurella haemolytica</i>	LktA protein (leukotoxin)	68%	50%
<i>Actinobacillus pleuropneumoniae</i>	RTX toxin determinant	68%	48%
<i>Escherichia coli</i>	Haemolysin - plasmid	58%	43%
<i>E. coli</i>	Haemolysin - chromosomal	58%	43%

Functional complementation by the *M. bovis* haemolysin

A construct which expressed the chromosomal-borne haemolysin of *E. coli* was obtained (pLG900; generated by combining the two plasmids pLG575 (26) and pLG816 (*hlyC* and *hlyA* cloned into pBluescriptSK). pLG900 comprises the four genes of the RTX operon, *hlyC*, *hlyA*, *hlyB*, *hlyD*, cloned

into pBluescriptSK and is capable of conferring a haemolytic phenotype on *E. coli* cells that were previously non-haemolytic. The A subunit (*hlyA*) of this construct was mutated such that it was no longer able to be expressed but the other genes involved in the operon (*hlyB*, *hlyC* and *hlyD*) remained intact.

- 5 The *E. coli* strain containing this construct (pLG900 / *hlyA* negative) was no longer haemolytic. However, the haemolytic phenotype was restored by providing *in trans* the cloned haemolysin subunit gene from *M. bovis* Dalton 2d (Figure 12). Thus it was confirmed that the cloned *M. bovis* haemolysin gene encoded a structural subunit that was most probably a member of the
- 10 RTX family of haemolytic enzymes.

Further sequence analysis has established that, like other members of the family, the *M. bovis* RTX A subunit gene is flanked by DNA sequences capable of encoding the RTX B,C and D proteins.

15 Conservation of the RTX A subunit among *M. bovis*

To determine whether the gene for the RTX A subunit was present in *M. bovis* strains representing the known pilus serotypes, a southern hybridisation analysis was performed using the coding region of the RTX A subunit as a probe.

- 20 Genomic DNA extracted from the seven serotype strains of *M. bovis* (15) was digested with *EcoRV* and separated using agarose gel electrophoresis. The DNA was transferred to a Hybond N+ filter (Amersham, Little Chalfont, Buckinghamshire, U.K.) using a previously described method (9). The probe used was a PCR amplified product that contained all of the
- 25 coding region from the A subunit of the RTX haemolysin of *M. bovis*. This fragment was labelled with $\alpha^{32}\text{P}$ -dATP using the Megaprime labelling system (Amersham, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturers instructions. High stringency conditions were used
- 30 (hybridisation temperature 68°C; 2 washes at room temperature in $2 \times \text{SSC} / 0.1\% \text{SDS}$; 1 wash at 68°C in $0.1 \times \text{SSC} / 0.1\% \text{SDS}$) and the resulting filters were exposed to autoradiographic film (Kodak, Rochester, New York, USA) for 5 to 24 hours before developing.

Figure 13 shows that the gene encoding the RTX A haemolysin subunit was conserved in all seven representative strains of *M. bovis* examined.

- 35 Interestingly, each of these strains is known to display the haemolytic phenotype on horse blood agar. In contrast, the non-haemolytic *M. bovis*

strain Gordon 26L3 did not hybridise to the RTX A gene probe possibly suggesting that *M. bovis* contains only a single structural gene responsible for the haemolytic phenotype detected on horse blood agar.

- 5 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this thirty-first day of August 1999

Commonwealth Scientific and
Industrial Research Organisation, The
University of Melbourne
Patent Attorneys for the Applicant:

F B RICE & CO

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SEQ. ID. NO. 1

Protease amino acid sequence:

```

1  mslqtqpakr gfyvkplasma cmlvisasst vsyansapmi vdsqynssky
51  sfydyyl dfl krfrptptpv pspvrpapel vrptpapipa ptpvptpapi
101 sggisgsyia pvspsevrqp dytrrvqanl krnqpapsag trtgysvmdt
151 snnsnltskf ygttedgyae rldnlkntid trqakvgvid tginrfnrld
201 vganvhdtqi ecvsagrste ytpendsgiv eipttsasgs hgnqmaavia
251 gnngmtnaki ygsdsidrrs nggnhflmmr klnqdhgvgi fnnswgsnnt
301 dqwydaqrl nynpttgqin pnpyrtsitn aevtlpvihd limnrdslii
351 katgneglnd ahdenlaplm nsnfkkgfit vsspredfgk anhcgrtaew
401 cvsatsstqn yandgrlssy kgtspatarv sgtavlvqsa ypwmknenis
451 qtilgtakdf seitanspng yqglrkvsrl psgyygsyyt dnqgnfyvpg
501 nvnwenrriv anhgknitw edgwglldpe aaakgyggfy wdnveldtkg
551 tplsvfyndl kgdkgftkkk egklvftggn sykgdsvieg gslevngnng
601 gstmvvkgge ltgygnvanv rqtggwvne glnnirgdyn intqrgvdag
651 lkaqfgnmlt vdggaklggt lnltgetkdg iisksgsrst vlrakrgleg
701 qfdnyrssnp lfevtnevtyt pevdrngrvv ggsrtndvq vtakrlsagn
751 vvygismnds gsrvaqnldk vlndldkkqe tqgsltsdek qfanrvftgf

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801 enmmsgaesk lstvstnrel ykldptfyad salnavedsa nhatefgkrv
851 saprgvwgni shhdydvele hatsarkgnn isvgastqta adisvgaqld
901 vskldleesv ygignkttkd sigltvgask klgdaylsgw vkgakvdtea
951 nrgensnkve yngklygagi qagtnidtas gvsvqpyafv nhqqykndgs
1001 fndglnvdd ieakqtqvgv gadmvfqatp alqltggvgv ahavsrtdnl
1051 dtryvgtatd vqygtwtdk tkwsakvgan ynvtpnsqvg lnysytgsgd
1101 sdasqvgvsf tskf

SEQ. ID. NO. 2

Protease DNA sequence: (Upstream and downstream sequence included)

```
1  ttctcatggt tgacagctta tcatcgataa gctttaatgc ggtagtttat
51  cacagttaaa ttgctaacgc agtcaggcac cgtgtatgaa atctaacaat
101  gcgctcatcg tcatcctcgg caccgtcacc ctggatgctg taggcatagg
151  cttggttatg ccggtactgc cgggcctctt gcgggatatc gtccattccg
201  acagcatcgc cagtcactat ggcgtgctgc tagcgctata tgcgttgatg
251  caatttctat gcgcaccgct tctcggagca ctgtccgacc gctttggccg
301  ccgcccagtc ctgctcgctt cgctacttgg agccactatc gactacgcga
351  tcatggcgac cacaccgctc ctgtggatca ataattaatg aacatatata
401  ctctatttaa tatttcttat ttattcgtaa tattgccata aaaataatac
451  attatttcta tattaactaa actgttaata tttgtaaata ataaacattt
501  gtttatctaa aaaaataaat aatataaatc aagcaattac aatcttattt
551  ttgaaaatac aataaactg caattgctta atctagacat taagtttatt
601  tttgattaaa attgccaaaa cttgtgtaaa taagtttcac cgaattgata
651  ctttaagggt atcaatattg caacatggta aatgattgct atgttggttg
701  gcattgcata aattgtctat aataacttgt tatggatgat tgatggcaat
751  gataaactta gtgacaatga taaacgcaaa gaggtgtaat atgtcattac
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951 attactatth agatttcctt aaacgtttta gaccaactcc aactccagtg
1001 ccaagccctg tgagaccggc tcctgaactc gttcgtccga cccagcccc
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1101 tatcaggtag ctatattgct ccagtatgc catcagaggt gagacagcct
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2051 aagggtacat cacctgcaac cgctcgtgtg tccggcacgg cagtgcctgt
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2151 tgggtactgc caaggatttc tcagagatta ctgccaattc acctaatggc
2201 taccaaggac taagaaaggt tagtagattg ccatctgggtt attacggctc
2251 ttattacact gacaatcagg gtaatttcta tgttcctggc aatgtcaatt
2301 gggaaaaccg tcgaattgtc gctaatacata acggcaagaa cattacatgg
2351 gaagatgggtt ggggtttggt agatccagaa gcggccgcta aaggttatgg
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2451 ctgtattcta caatgaccta aaaggtgata aaggctttac caaaaaaggt
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2951 ccagaagtag acagaaatgg cagagtggta ggtggttcac gcacgaacaa
3001 tgacgtgcaa gtaactgcca aacgtctaag tgcaggcaat gttgtttatg
3051 gcatcagcat gaatgacagt ggtagccgtg ttgcacaaaa cctagacaaa
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3251 tacaagcttg acccaacttt ctatgctgac agtgcattaa acgcagtaga
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3351 gaggtgtttg gggtaatatc agtcaccatg attatgatgt agaactagag
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3551 agcattggct tgactgttgg tgcttctaag aagttgggtg atgcctatct
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3651 aaaactctaa caaagttgag tacaatggta agctatatgg tgctggtatc
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3751 tgcctttgtt aaccatcagc agtacaaaaa cgatggtagc ttcaatgacg

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4201 aaaattttcc caaaaaagc gtgataatta ccacgctttt ttattgcata
4251 ttgcaaaata gtattgcatt tatgggttgt taagcaaccc gtccaaatac
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4351 cgtcaatgtg tcggcatcat caaccattac cgac

SEQ. ID. NO. 3

Lipase protein sequence:

1 mkksafakys alalmvgmcl htayakefsq viifgdsld tgrlkdmvar
51 kdgtlgnltlq psfttnpdpv wsslfaqsyg ktasantpyn ptgtnyavgg
101 arsgsevnwn gfvnvpstkt qitdhltatg gkadpntlya iwigsndlis
151 asqatttaea qnaikgavtr tvidietlnq agattilvpn vpdlsltpra
201 iygeslmagv qdkaklassl ynsglfealn qstaniipan tfallqeatt
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351 drlsgsqhsi wanvhasdrt dpttqigldv agssshtgay lshqnqdyvl
401 ddtlssdvkt igmglyhrhd ignvrlkgva gidrlsvdth rhidwegasr
451 shtadttarr fhaglqasyg idmgkatvrp ligvhaqkvk vrdlvenept
501 lstamrfgeq eqkslqgeig vdwaypispal tlttggiaha hefndderti
551 natltsirey tkgfntsvst dkshattahl gvqqqlgkan ihagvhathq
601 dsdtdvggsl gvrlmf

SEQ. ID. NO. 4

Lipase DNA sequence: (Upstream and downstream sequence included)

```
1  tgacaaataa ttgggcattg ggcagataac ccatcaaaga cccaaagcaa
51  ccataaatc aaaaaaacac ttgtaatttt tgtaatatct tgttacactt
101 tacaagtgtt ttacttttga aagcaactca gagagtaata atgaaaaaat
151 ccgcctttgc caaataactca gcacttgccc taatgggttg gatgtgcctg
201 cacaccgctt acgccaagga gtttagccaa gtcatcattt ttggggacag
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601 ccaccacaac agccgaagcc caaaacgcca ttaaagggtgc ggtaactcgc
651 accgtgatag acatcgaaac actcaatcaa gcaggggcga caaccatttt
701 ggtgccaaat gtgcctgatt tgagcctcac gccccgagcc atctatggcg
751 aaagcctcat ggcaggcgtg caagacaaag ccaaactcgc ctcaagtctg
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801 tataatagcg gtctgtttga agcattaaat caatccaccg ccaacatcat
851 ccctgccaac acctttgccc tactccaaga agcgaccaca aataaagaag
901 cctttggttt taaaaacacg caaggcgtgg cgtgtcaaat gcccgctcgt
951 accacagggg cggtatgatgt ggcttctact tccttggcat gtaccaaagc
1001 caatcttata gaaaacgggg caaatgacac ctacgccttt gccgatgaca
1051 ttcacccatc gggacgcacg caccgcattt tggcacagta ttaccgttct

1101 atcatggacg ccctactca catgggtaaa ctctcaggcg agcttgtcaa
1151 aacaggttca gccacgacc gtcattgtta ccgtcagctt gacaggctta
1201 gtggetcaca gcacagcatt tgggcaaacg tccatgccag cgaccgtacc
1251 gacccacca cccaaatcgg cttggacgtg gcaggttcat caagccatac
1301 aggggcgtat ctgagccacc aaaaccaaga ttatgtgctg gatgacacc
1351 tatcatcaga tgtcaaaacc attggcatgg ggctgtatca tcgccatgac
1401 atcggcaatg tccgtctaaa aggcgtggca ggtatcgacc gacttagcgt
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2101 caaacatgag

SEQ. ID. NO. 5

RTX A subunit amino acid sequence:

1 msninviksn iqaglnstks glknylaip kdydpqkggt lndfikaade
51 lgialrlaep nhtetakksv dtvnqflslt qtgiaisatk lekflqkhst
101 nklakgldsv enidrkkgka snvlstlssf lgtalagiel dslikkgdaa
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201 nklqnlfnfsk tnlgleiitg llsgisagfa ladknastgk kvaagfeln
251 qvignvtkai ssyvlaqrva aglsttgava alitssimla isplafmnaa
301 dkfnhanald efakqfrkfg ydgdhllaey qrgvgtieas lttistalga
351 vsagvsaaav gsavgtplal lvagvtglis gileaskqam fesvanrlqg
401 kilewekqng gqnyfdkgyd sryaaylann lkflselnke leaerviait
451 qqrwdnnige lagitklger iksgkayada fedgkkveag snitldaktg
501 iidisnsngk ktqalhftsp lltagtesre rltngkysyi nklkfgrvkn
551 wqvtdgeass kldfskviqr vaetegtdei glivnakagn ddifvgqgkm
601 nidggdghdr vfyskdggfg nitvdgtsat eagsytnrk vargdiyhev
651 vkrqetkvk rtetiqyrdy elrkvggyq stdnlksvee vigsqfndvf
701 kgskfndifh sgegddlldg gagddrlfgg kgndrlsgde gddlldggsg
751 ddvlnngagn dvyifrkgdg ndtlydgtgn dklafadani sdimiertke

801 giivkrndhs gsiniprwyi tsnlqnyqsn ktdhkieqli gkdgsyitsd

851 qidkilqdkk dgtvitsqel kkladenksq klsasdiass lnklvgsmal

901 fgtansvssn alqpitqptq gilapsv

SEQ. ID. NO. 6

RTX A subunit DNA sequence: (Upstream and downstream sequence included)

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1  atgagaacgt tattttcaga tgaattgttt agagcgattc gtgtagatgg
51  aaattcatcg catggtaaga tatctgaatt ttatggaaag tctgttgatt
101 caaaattagc ctcaagaata ttgcacaat atcacgaaga ttgacgagc
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551 tagacagtgt agaaaatatt gatcgtaaag taggtaaagc aagtaatgta
601 ttatcaacat taagctcttt ttgggcact gcattagcgg gtatagaact
651 tgattcttta atcaaaaaag gtgatgctgc acctgatgct ttggetaaag
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751 caaacgattg aagcattttc ttcacagtta gcaaagttag gttctactat
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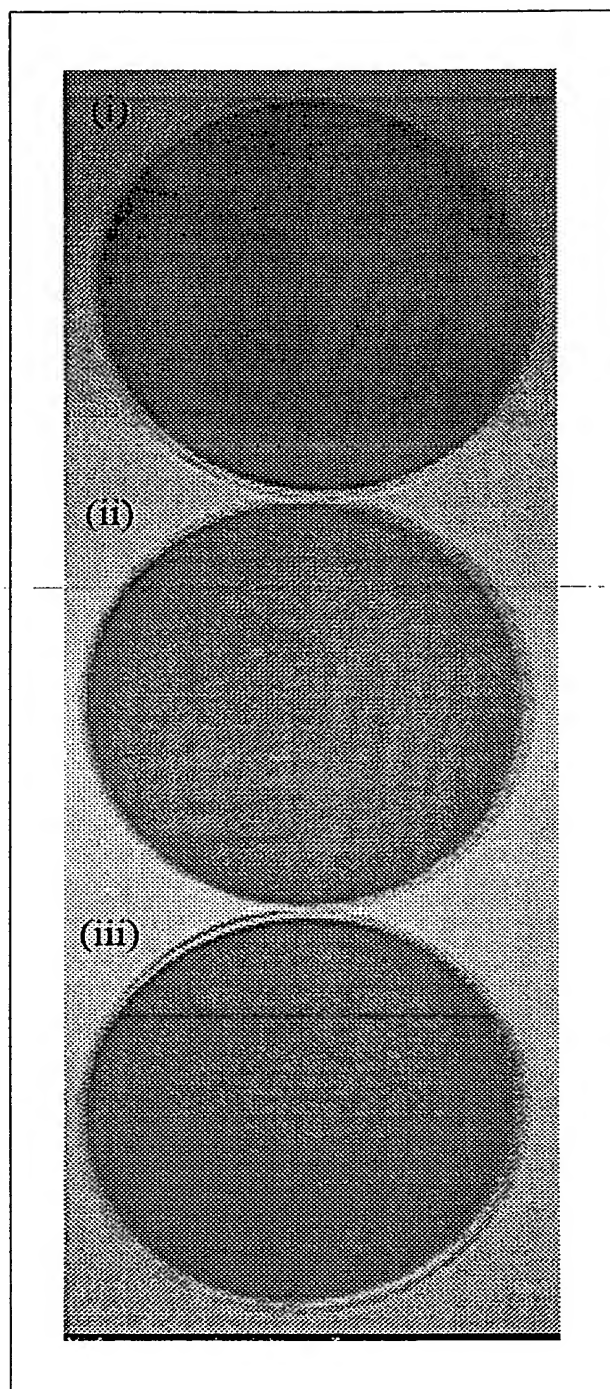


Figure 1: Phenotype of protease from Dalton 2d. (Cultured on skim milk agar)

- (i) Dalton 2d
- (ii) pJF1/DH5 α
- (iii) pHc79/DH5 α

1 ttctcatgtt tgacagctta tcatcgataa gctttaatgc ggtagtttat cacagttaaa ttgctaacgc agtcaggcac cgtgtatgaa
91 atctaacaat gcgctcatcg tcatcctcgg caccgtcacc ctggatgctg taggcatagg cttggttatg ccggtactgc cgggcctctt
181 gcgggatata gtccattccg acagcatcgc cagtcaatat ggcggtgctg tagcgctata tgcgttgatg caatttctat gcgcaccggt
271 tctcggagca ctgtccgacc gctttggccg ccgcccagtc ctgctcgctt cgctacttgg agccactatc gactacgcga tcatggcgac
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541 aatcttattt ttgaaaatac aataatactg caattgctta atctagacat taagtttatt ttgtattaaa attgccaaaa cttgtgtaaa
631 taagtttcac cgaattgata ctttaagggt atcaatatgt caacatggta aatgattgct atgttgttgg gcattgcaga aatgttctat
721 aataacttgt tatggatgat tgatggcaat gataaactta gtgacaatga taaacgcaaa gaggtgtaat atgtcattac aaactcaacc
1 M S L Q T Q
811 tgccaagaga gggttctatg ttaagccttt aagtatggct tgcattgctg taattagtgc tagtagtacg gtaagttatg ccaactcagc
7 P A K R G F Y V K P L S M A C M L V I S A S S T V S Y A N S
901 tccaatgatt gttgattcac agtacaatag ttctaataac tctttctacg attactattt agatttccct aaacgtttta gaccaactcc
37 A P M I V D S Q Y N S S K Y S F Y D Y Y L D F L K R F R P T
991 aactccagtg ccaagccctg tgagaccggc tctgaactc gttcgtccga cccagccccc gattccggct ccaacgcctg tgccaacacc
67 P T P V P S P V R P A P E L V R P T P A P I P A P T P V P T
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97 P A P I S G G I S G S Y I A P V S P S E V R Q P D Y T R R V
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127 Q A N L K R N Q P A P S A G T R T G Y S V M D T S N N S N L
1261 atctaatttt tatggcacia ccgaagatgg ttatgccgag cgtcttgaca acctaaagaa caccattgat acacgtcaag ccaaagtagg
157 T S K F Y G T T E D G Y A E R L D N L K N T I D T R Q A K V
1351 tgtgattgat acaggcatta accgcttcaa ccgagacttg gttggtgcaa atgtgcatga tacacagatt gagtgtgttt ctgctggacg
187 G V I D T G I N R F N R D L V G A N V H D T Q I E C V S A G
1441 ttccacctgc tatacgccag aaaatgatcc aggcattggt gaaatcccaa caacctctgc tagtggtagt catggcaacc aaatggcggc
217 R S T C Y T P E N D S G I V E I P T T S A S G S H G N Q M A
1531 tgtatcgctt ggtaacaacg gcatgaccaa cgccaaaatt tacggcagtg acagtattga tgcagcttca aatgggtggc accatttctt
247 A V I A G N N G M T N A K I Y G S D S I D R R S N G G N H F
1621 gatgatgcgt aagctgaacc aagaccatgg tgtcaagatt tttacaact cttggggttc taacaacact gaccaatggt actacgatgc
277 L M M R K L N Q D H G V K I F N N S W G S N N T D Q W Y Y D
1711 tcagcgccta aattacaatc ctactacagg acagattaat ccaaatcctt acagaaccag tattaccaat gctgaagtga ctttgcctgt
307 A Q R L N Y N P T T G Q I N P N P Y R T S I T N A E V T L P
1801 cattcatgat cttattatga atcgtgactc gcttatcatt aaagcaacag gtaacgaagg cttgaacgat gctcatgatg aaaacctagc
337 V I H D L I M N R D S L I I K A T G N E G L N D A H D E N L
1891 accgctcatg aacagcaact tcaaaaaagg ttctattact gtctcctcgc ctagagaaga ttttggtaaa gcgaatcatt gtggtcgaac
367 A P L M N S N F K K G F I T V S S P R E D F G K A N H C G R
1981 tgccgaatgg tgtgtatccg caacatcacc tacccaaaat tacgccaacg atggcagact gagtagctat aagggtacat cacctgcaac
397 T A E W C V S A T S S T Q N Y A N D G R L S S Y K G T S P A
2071 cgctcgtgtg tccggcacgg cagtgtcgtg gcaatctgct tatccttgga tgaataatga aaatatctct caaacctttt tgggtactgc
427 T A R V S G T A V L V Q S A Y P W M K N E N I S Q T I L G T
2161 caaggatttc tcagagatta ctgccaatcc acctaatggc taccaaggac taagaaagggt tagtagattg ccactctggtt attacggctc
457 A K D F S E I T A N S P N G Y Q G L R K V S R L P S G Y Y G
2251 ttattacact gacaatcagg gtaatttcta tgttcctggc aatgtcaatt gggaaaaccg tcgaattgtc gctaatacata acggcaagaa
487 S Y Y T D N Q G N F Y V P G H V N W E N R R I V A N H N G K
2341 cattacatgg gaagatggtt ggggtttgtt agatccagaa gcggccgcta aaggttatgg tggtttctat tgggataatg tggaaattaga
517 N I T W E D G W G L L D P E A A A K G Y G G F Y W D N V E L
2431 cactaaaggc acgcttttat ctgtattcta caatgacctt aaagggtgata aaggctttac caaaaaagggt gaaggtaaac ttgtctttac
547 D T K G T P L S V F Y N D L K G D K G F T K K G E G K L V F
2521 tggtaataat agctataaag gcgactctgt catcgagggt ggttcactag aagtaaatgg taacaacggt ggttcaacca tggttgttaa
577 T G N N S Y K G D S V I E G G S L E V N G N N G G S T M V V
2611 aggtggtgaa ctaacagggt atggtaatgt agctaattgt cgtaaacag gtggttggtt taacaacgaa ggtaacctaa acatcagagg
607 K G G E L T G Y G N V A N V R Q T G G W V N N E G N L N I R

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2701  tgactacaac atcaacactc aacgtggcgt ggatgctggt ctaaaagctc aatttggcaa catgcttacc gtggacggta aggccaaact
637  G D Y N I N T Q R G V D A G L K A Q F G N M L T V D G K A K

2791  aggtggtaca ctaaactctaa ctggtgagac caaagatggt atcatcagca aatcaggtag ccgtagcact gtacttcgtg ctaagcgttg
667  L G G T L N L T G E T K D G I I S K S G S R S T V L R A K R

2881  tcttgaaggt caatttgaca attatcggtc aagcaaccca ttatttgaag taacaaatgt tgaatatacg ccagaagtag acagaaatgg
697  G L E G Q F D N Y R S S N P L F E V T N V E Y T P E V D R N

2971  cagagtggta ggtggttcac gcacgaacaa tgacgtgcaa gtaactgcca aacgtctaag tgcaggcaat gttgtttatg gcacgagcat
727  G R V V G G S R T N N D V Q V T A K R L S A G N V V Y G I S

3061  gaatgacagt ggtagccgtg ttgcacaaaa cctagacaaa gtacttaatg atttagataa aaaacaagaa acacaagggt cactgaccag
757  M N D S G S R V A Q N L D K V L N D L D K K Q E T Q G S L T

3151  tgatgagaag caatttgcta accgtgtatt cactggtttt gaaaacatga attctggtgc agaactctaaa ctttctacag taagcaccaa
787  S D E K Q F A N R V F T G F E N M N S G A E S K L S T V S T

3241  ccgtgagcta tacaagcttg acccaacttt ctatgctgac agtcatttaa acgcagtaga agacagtgtc aaccatgcaa ccgaatttgg
817  N R E L Y K L D P T F Y A D S A L N A V E D S A N H A T E F

3331  taagcgtggt agcgccccaa gaggtgtttg gggtaatatc agtcaccatg attatgatgt agaactagag catgctacaa gtgcacgtaa
847  G K R V S A P R G V W G N I S H H D Y D V E L E H A T S A R

3421  aggcaacaac attagtgttg gtgcaagcac tcaaaactgca gccgacatta gtgttggtgc acaacttgat gtaagtaaac ttgacttgga
877  K G N N I S V G A S T Q T A A D I S V G A Q L D V S K L D L

3511  agaactctgt tatgttattg gcaacaaaaa caaaactgac agcattggct tgactgttgg tgcttctaag aagttgggtg atgcctatct
907  E E S V Y G I G N K T K T D S I G L T V G A S K K L G D A Y

3601  atcaggttgg gtaaaagggt ccaaagttga tacagaagcg aacgtggtg aaaactctaa caaagttgag tacaatggta agctatatgg
937  L S G W V K G A K V D T E A N R G E N S N K V E Y N G K L Y

3691  tgctggtatc caagcgggta caaacattga tactgcatcg ggcgtgagtg tacaacctta tgcctttgtt aaccatcagc agtacaaaaa
967  G A G I Q A G T N I D T A S G V S V Q P Y A F V N H Q Q Y K

3781  cgatggtagc ttcaatgacg gtcttaacgt tgttgacgac atcgaagcaa acaaaactca ggtagggttg ggtgctgata tgggtgtcca
997  N D G S F N D G L N V V D D I E A K Q T Q V G V G A D M V F

3871  agcaaacact gctctacagc ttactggttg tgtgcaagtt gctcagcgtg ttagcgtga caccaaccta gacactcgct atgttggtag
1027  Q A T P A L Q L T G G V Q V A H A V S R D T N L D T R Y V G

3961  agcgacagat gtacagtatg gcacttggga tactgacaaa accaaatggt cagccaaggt tgggtgtaac tataatgtga caccaaacag
1057  T A T D V Q Y G T W D T D K T K W S A K V G A N Y N V T P N

4051  ccaagtgggt ctaaaactaca gctacacagg tagtggcgat tcagatgctt ccaaagtggt tgtgagcttc accagcaagt tctaattcat
1087  S Q V G L N Y S Y T G S G D S D A S Q V G V S F T S K F

4141  taataaggca acaaaaaaca gcacaatttc ggttgtgctg ttttttgtga tgccgagcgt aaaattttcc caaaaaaagc gtgataatta

4231  ccacgctttt ttattgcata ttgcaaaata gtattgcatt tatgggtgtg taagcaaccc gtccaaatac cccctaaaca actccacccc

4321  aatcggtgct aactgtttt gccacaggct cgtcaatgtg tcggcatcat caaccattac cgac

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Figure 2: Nucleotide and amino acid sequence of a protease from Dalton 2d.

Putative promoter sequence singly underlined.

Putative ribosome binding site shown in bold and underlined.

Putative start codon shown in bold.

Putative transcription terminator sequences indicated by inverted arrows.

Numbering for both the nucleotide and amino acid sequences are shown down the left hand-side.

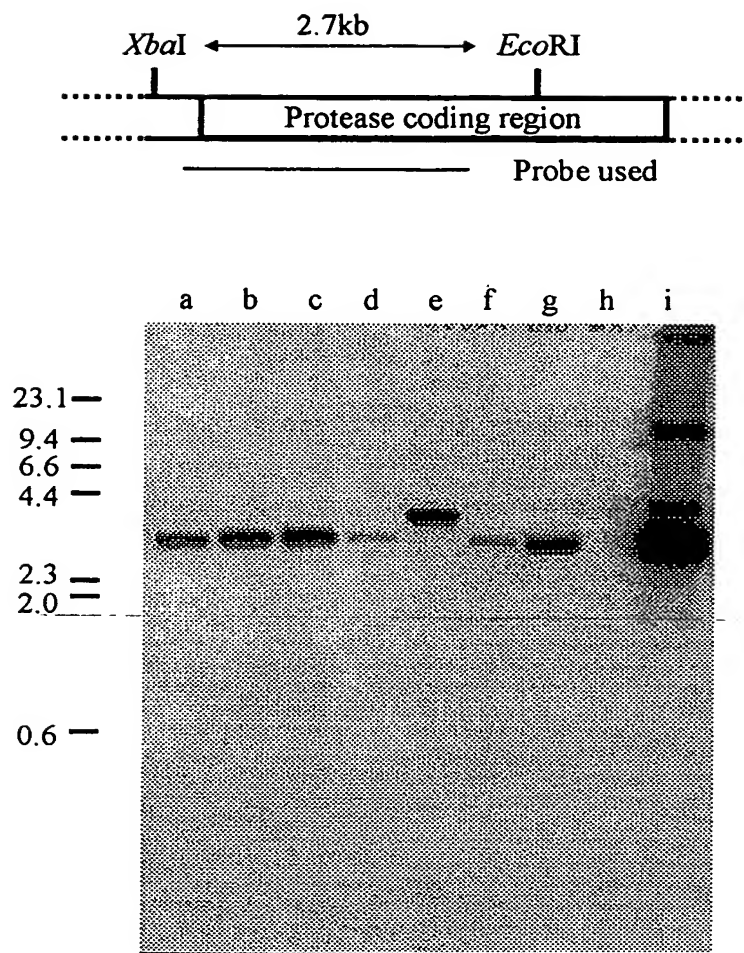


Figure 3: Southern hybridisation carried out to determine whether the protease cloned from Dalton 2d was conserved across all seven serotypes of *M. bovis*. Shown above the southern blot is a diagram of the sites used in the genomic digests and the area to which the probe should bind.

Lanes:

- a. S276R
- b. 3WO7
- c. Dalton 2d
- d. R593L
- e. Tat849
- f. 218R
- g. Fleur462
- h. Negative control
- i. pJF1 digested with *Xba*I and *Eco*RI

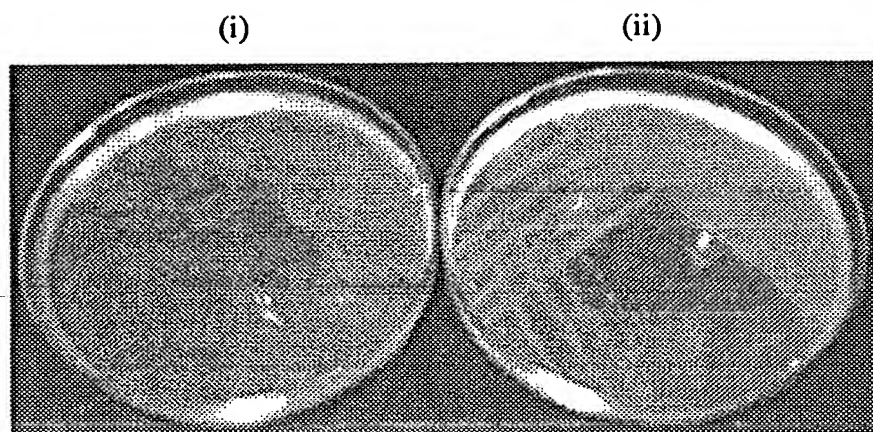


Figure 4: Phenotype of a lipase from *M. bovis*. (Cultured on Tween 80 media)

(i) pBR322 / MC1061

(ii) pMB1 / MC1061^{ts}

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1  tgggcagata acccatcaaa gacccaaagc aacccataaa tcaaaaaaac acittgtaatt tgtgtaatat cttgttacac ttacaaagtg
91  tttttacttt gaaagcaact cagagagtaaa taatgaaaaa atccgccttt gccaaatact cagcacttgc cctaattggtt gggatgtgcc
1  M K K S A F A K Y S A L A L M V G M C
181 tgcacaccgc ttacgccaag gagtttagcc aagtcacatc ttttggggac agcttggtccg atacaggctg cctaaaagat atggctgccc
20 L H T A Y A K E F S Q V I I F G D S L S D T G R L K D M V A
271 gaaaagatgg cacccttggc aacaccttac agccatcttt taccaccaac cccgaccctg tatggtcaag cttatttggc caaagttatg
50 R K D G T L G N T L Q P S F T T N P D P V W S S L F A Q S Y
361 gcaaaaccgc cagtgcaca acgccttaca atccacttgg cactaactat gccgtgggag gagctcgctc tggctcgag gtcaattgga
80 G K T A S A N T P Y N P T G T N Y A V G G A R S G S E V N W
451 atggttttgt gaatgtacc tccacaaaa cgcaaatcac cgaccatttg accgcccagc gtggcacaag cgaccctaac accctgtatg
110 N G F V N V P S T K T Q I T D H L T A T G G K A D P N T L Y
541 ccatttggat tggctcta gacttaattt cagcttctca agccaccaca acagccgaag cccaaaaccg cattaaaggc gcggttaactc
140 A I W I G S N D L I S A S Q A T T T A E A Q N A I K G A V T
631 gcaccgtgat agacatcgaa acactcaatc aagcaggggc gacaaccatt ttggtgcaa atgtgcctga tttgagctc acgccccgag
170 R T V I D I E T L N Q A G A T T I L V P N V P D L S L T P R
721 ccattctatg cgaagcctc atggcaggcg tgcaagada agccaaactc gcctcaagtc tgtataatag cggctgtgtt gaagcattaa
200 A I Y G E S L M A G V Q D K A K L A S S L Y N S G L F E A L
811 atcaatccac cgccaacatc atccctgcca acacctttgc cctactccaa gaagcgacca caaataaaga agcctttggt tttaaaaaca
230 N Q S T A N I I P A N T F A L L Q E A T T N K E A F G F K N
901 cgcaaggcgt ggctgtcaa atgcccgtc gtaccacagg ggcgatgat gtggttcta cttccttggc atgtacaaa gccaatctta
260 T Q G V A C Q M P A R T T G A D D V A S T S L A C T K A N L
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991 tagaaaacgg ggcaaatgac acctacgcct ttgccgatga cattcaccca tggggagcca cgcaccgcat tttggcacag tattaccgtt
290 I E N G A N D T Y A F A D D I H P S G R T H R I L A Q Y Y R
1081 ctatcatgga cgccctact cacatgggta aactctcagg cgagcttgtc aaaacagggt cagcccagca ccgtcatgtt tacctgcagc
320 S I M D A P T H M G K L S G E L V K T G S A H D R H V Y R Q
1171 ttgacaggct tagtggtc cagcacagca tttgggcaaa cgtccatgcc agcgaccgta ccgacccac caccaaaatc ggcttggagc
350 L D R L S G S Q H S I W A N V H A S D R T D P T T Q I G L D
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380 V A G S S S H T G A Y L S H Q N Q D Y V L D D T L S S D V K
1351 ccattggcat ggggtgtat catcgccatg acatcgccaa tgtccgtcta aaaggcggtg caggatcgca ccgacttagc gtggatacgc
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1441 accgcatat cgactgggag ggggcaagcc gttcgacac ggcagacacc accgcccagc gttttcatgc agggctacaa gccagctatg
440 H R H I D W E G A S R S H T A D T T A R R F H A G L Q A S Y
1531 gcatagacat gggcaaaagc accgtgcgtc cgcttatcgg cgtacatgcc caaaaagtca aatgctgtga tttggtagag aatgagccta
470 G I D M G K A T V R P L I G V H A Q K V K V R D L V E N E P
1621 ccctatccac cgccatgcgt tttggcgagc aagaacaaaa gtcctacaa ggcgagattg gcgtcatgtt ggcttatccg attagccctg
500 T L S T A M R F G E Q E Q K S L Q G E I G V D V A Y P I S P
1711 ctttgactct gacggggcgt atcgtcacg ctcatgagtt taacgatgat gaacgcacca ttaatgccac ttaacctcc attcgtgaat
530 A L T L T G G I A H A H E F N D D E R T I N A T L T S I R E
1801 acacgaaggg cttaataca agcgttagca ccgacaaatc tcacgccacc accgctcatc tggcggtaca agggcaactt ggcaaggcaa
560 Y T K G F N T S V S T D K S H A T T A H L G V Q G Q L G K A
1891 atattcatgc aggcgttcac gccaccacc aagacagcga tacagacgtg ggtgggtcgc ttggggttcg cttgatgttt tgattggctt
590 N I H A G V H A T H Q D S D T D V G G S L G V R L M F -
1981 ttaaagataa aaagtggat catgccactt tttattttgc caaaaatcta tgtttgagta catcaaagcc tttcacatca tcgcatcg
2071 atgataagct gtcaaacatg ag

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Figure 5: Nucleotide and amino acid sequence of a lipase from Dalton 2d.
 Putative promoter sequence singly underlined.
 Putative ribosome binding site shown in bold and underlined.
 Putative start codon shown in bold.
 Putative transcription terminator sequences indicated by inverted arrows.
 Numbering for both the nucleotide and amino acid sequences are shown down the left hand side.

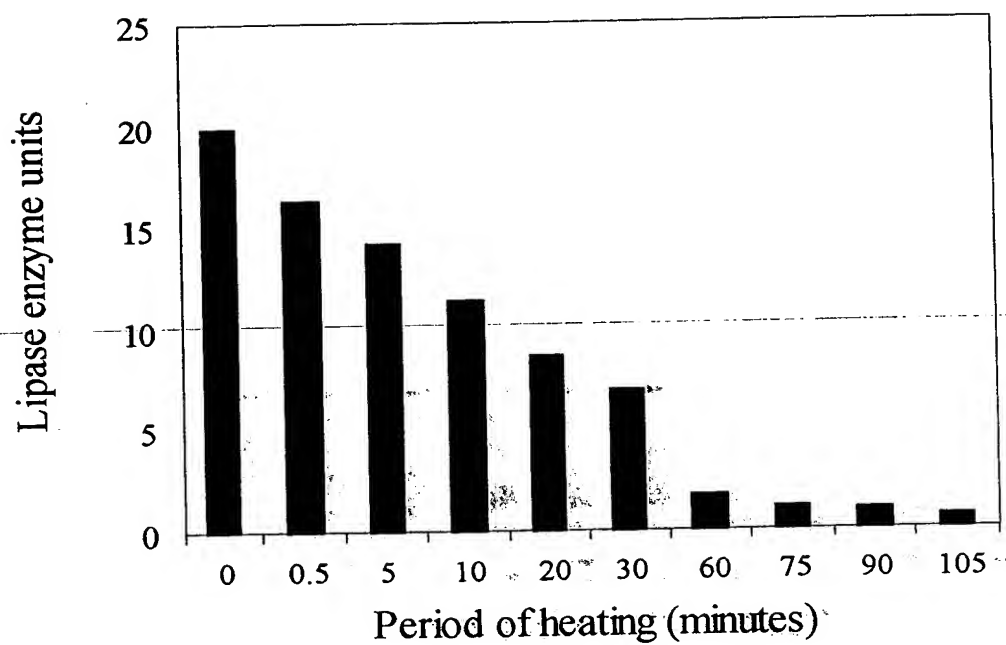
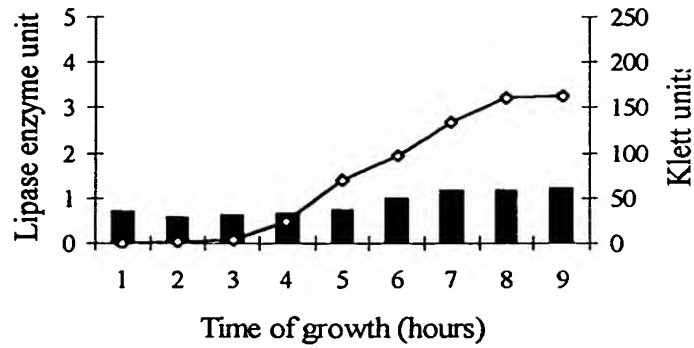


Figure 6: Heat stability of the lipase from *M. bovis* when expressed in its recombinant form (pMB1 / MC1061). (Heating carried out at 90°C)

(i) Dalton 2d / *M. bovis*



(ii) pMB1 / MC1061

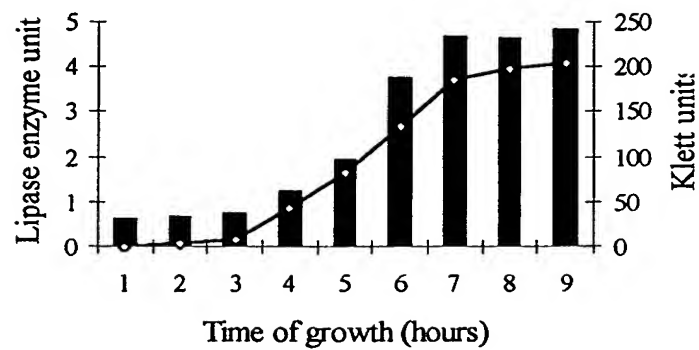


Figure 7(a): Comparison of the growth rate and expression levels of the lipase of *M. bovis* when in its (i) native form and (ii) recombinant form. The growth rate is shown as solid bars and the lipase expression levels as open diamonds.

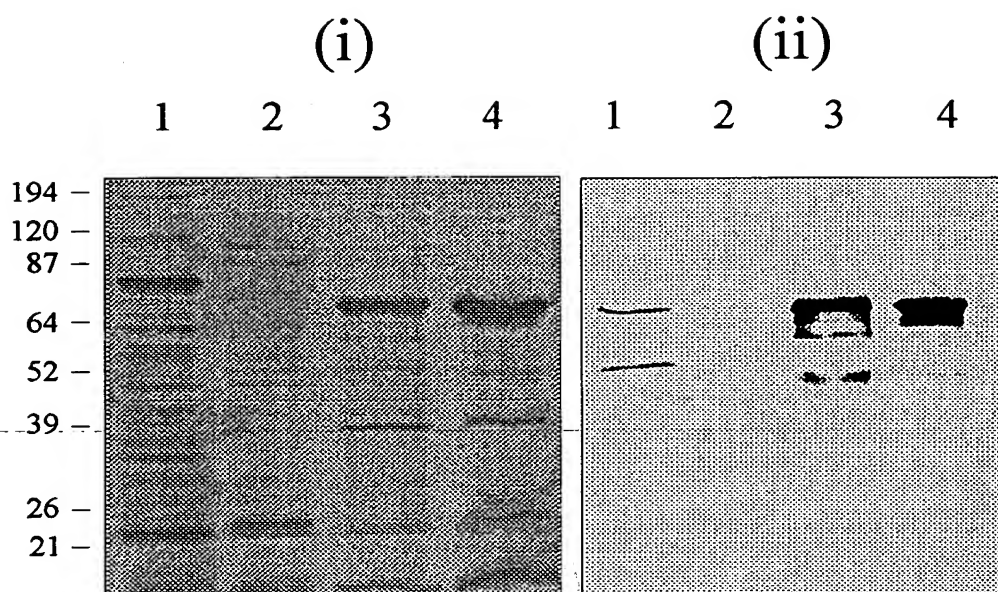


Figure 7(b): (i) Coomassie stained SDS-PAGE gel (ii) Western blot probed with antisera to the recombinant heat deactivated lipase. Demonstrates the expression levels of the recombinant form of the lipase in *E. coli* compared to those observed in the parent strain Dalton 2d. (ammonium sulfate precipitated supernatants)

Lanes: 1 – Dalton 2d

2 – pMB5 (lipase negative clone)

3 – pMB1 (lipase positive clone – 5.4kb insert)

4 – pMB4 (lipase positive clone – 2.1kb insert)

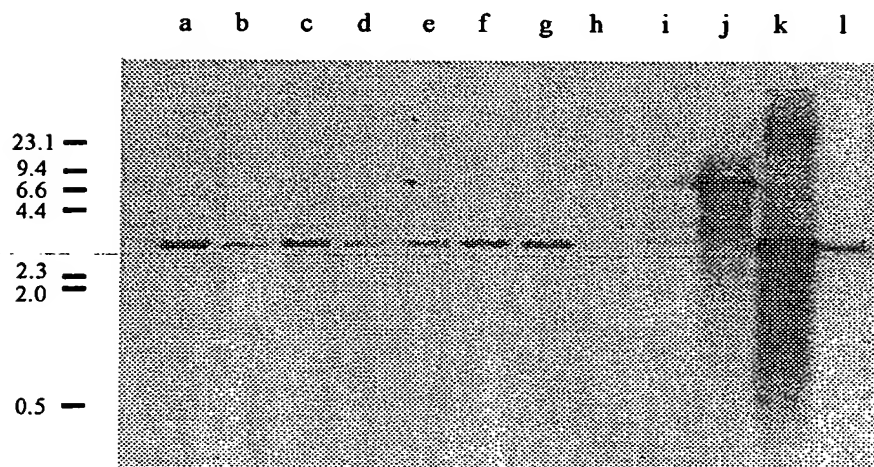
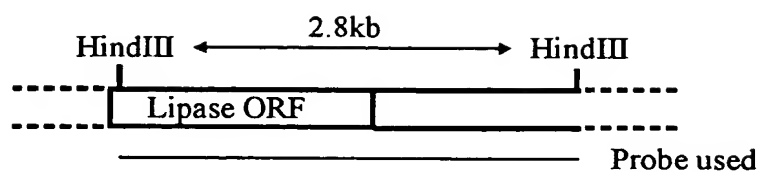


Figure 8: Southern blot analysis of the serotype strains of *M. bovis*, looking for the presence of a lipase gene. Genomic DNA was digested with *Hind*III and probed with the 2.8kb *Hind*III fragment which incorporates the majority of the lipase coding region from *M. bovis* strain, Dalton 2d.

- Lanes:
- a. S276R
 - b. 3WO7
 - c. Dalton 2d
 - d. R593L
 - e. Tat849
 - f. 218R
 - g. Fleur462
 - h. space
 - i. pBR322
 - j. pMB4 (lipase positive clone – smaller insert than pMB1)
 - k. pMB1
 - l. Dalton2d.

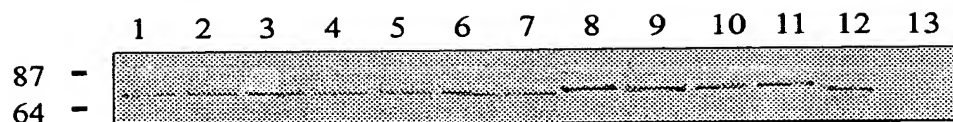


Figure 9: Western blot of the serotype strains of *M. bovis* to investigate whether all strains express the lipase gene cloned from Dalton 2d. Whole cell preparations were separated on SDS-PAGE and transferred to nitrocellulose, this was probed with the recombinant lipase antisera.

- Lanes:
1. S276R
 2. 3WO7
 3. Dalton 2d
 4. R593L
 5. Tat849
 6. 218R
 7. Fleur462
 8. Q220 (United Kingdom isolate)
 9. Epp63 (United States isolate)
 10. Gordon 26L3 (non-haemolytic strain)
 11. UQV148NF (haemolytic strain)
 12. pMB1 / MC1061 (lipase positive clone)
 13. pMB5 / MC1061 (lipase negative clone)

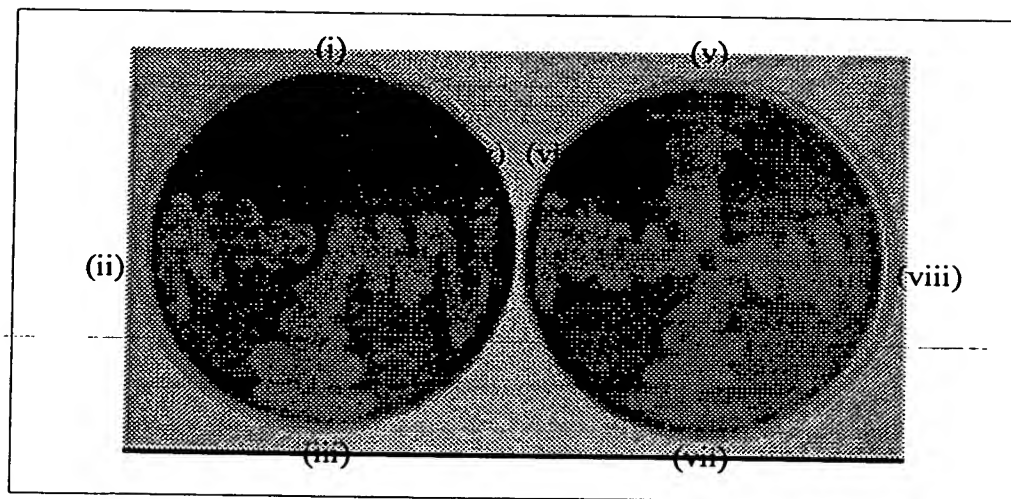


Figure 10: Activity displayed by *M. bovis* serotype strains when cultured on horse blood agar plates for 24 hours at 37°C followed by refrigeration at 4°C.

Strains:	(i)	DH5α / <i>E. coli</i>
	(ii)	S276R
	(iii)	3WO7
	(iv)	Dalton 2d
	(v)	R593L
	(vi)	Tat849
	(vii)	218R
	(viii)	Fleur462

1 atgagaacgt tattttcaga tgaattgttt agagcgattc gtgtagatgg aaattcatcg catggtaaga tatctgaatt ttatggaaaag
M R T L F S D E L F R A I R V D G N S S H G K I S E F Y G K
91 tctgttgatt caaaattagc ctcaagaata ttgcacaaat atcacgaaga ttgacgagc aaattgtcaa ctcagaataa ttttattata
S V D S K L A S R I F A Q Y H E D L T S K L S T Q N N F I I
181 tctaaagata attaatacaa ccttttctaa cacaacgagg agagacatat tatgtccaat ataatgttaa ttaaatctaa tattcaagca
1 S K D N - M S N I N V I K S N I Q A
271 ggcttgaatt caacaaagtc tggattaaaa aatctttact tggctattcc caaagattat gatccgcaaa aaggtgggac tttaaatgat
14 G L N S T K S G L K N L Y L A I P K D Y D P Q K G G T L N D
361 tttattaaag ctgctgatga attaggtatt gctcgtttag cagaagagcc taatcacact gaaacagcaa aaaaatctgt tgacacagta
44 F I K A A D E L G I A R L A E E P N H T E T A K K S V D T V
451 aatcagtttc tctctctcac acaaaactgg attgctattt ctgcaacaaa attagaaaag ttcttaca aaacattctac caataagta
74 N Q F L S L T Q T G I A I S A T K L E K F L Q K H S T N K L
541 gccaaagggt tagacagtgtag aaaaaatatt gatcgtaaat taggtaaagc aagtaatgta ttatcaacat taagctcttt ttggggcact
104 A K G L D S V E N I D R K L G K A S N V L S T L S S F L G T
631 gcattagcgg gtatagaact tgattcttta atcaaaaaag gtgatgctgc acctgatgct ttggctaaag ctagtattga cttgattaat
134 A L A G I E L D S L I K K G D A A P D A L A K A S I D L I N
721 gagataattg gtaatctatc tcagagtact caaacgattg aagcattttc ttcacagtta gcaagtttag gttctactat atcgaggct
164 E I I G N L S Q S T Q T I E A F S S Q L A K L G S T I S Q A
811 aaaggcttct ctaatatagg aaacaagttg caaaacttaa atttttctaa aacaaatctt gggttggaaa taattactgg ttgctatca
194 K G F S N I G N K L Q N L N F S K T N L G L E I I T G L L S
901 ggcatcttcg caggctttgc tttagcggat aaaaatgcat cgactggcaa aaaagttgct gcagggtttg aattaagcaa tcaagttatt
224 G I S A G F A L A D K N A S T G K K V A A G F E L S N Q V I

991 ggtaaatgta caaaagcaat ttcttcatat gttttagcac aacgtgttgc tgctgggtcta tcaactactg gtgctgttgc tgctttaatt
254 G N V T K A I S S Y V L A Q R V A A G L S T T G A V A L I
1081 acttcatcga ttatgttggc aattagtcct ttggcattta tgaatgcagc agataaattc aatcatgcta atgcttctga tgagtttgca
284 T S S I M L A I S P L A F M N A A D K F N H A N A L D E F A
1171 aaacaattcc gaaaatttgg ctatgatggg gatcatttat ttggctgaata tcagcgtggg gtgggtacta ttgaagcttc attaaacta
314 K Q F R K F G Y D G D H L L A E Y Q R G V G T I E A S L T T
1261 attagtcagg cattaggtgc agtcttctgct ggtgtttccg ctgctgctgt aggatctgct gttggtacac cgattgcact attagttgca
344 I S T A L G A V S A G V S A A A V G S A V G T P I A L L V A
1351 ggtgttacag gattgatctc tggattttta gaagcgtcta aacaggcaat gtttgaagt gttgctaacc gtttacaagg taaaatttta
374 G V T G L I S G I L E A S K Q A M F E S V A N R L Q G K I L
1441 gagtgggaaa agcaaaatgg cggtcagaac tattttgata aaggctatga ttctcgttat gctgcttatt tagctaataa cttaaaattt
404 E W E K Q N G G Q N Y F D K G Y D S R Y A A Y L A N N L K F
1531 ttgtctgagc taaataaaga gttggaagct gaacgtgtta ttgcaatcac ccaacaacgt tgggataata atattgggta gttagcagg
434 L S E L N K E L E A E R V I A I T Q Q R W D N N I G E L A G
1621 attaccaaat tgggtgaacg cattaagagc ggaagagctt atgcagatgc ttttgaagat ggcaagaaaag ttgaagctgg ttccaatatt
464 I T K L G E R I K S G K A Y A D A F E D G K K V E A G S N I
1711 actttggatg ctaaaactgg tatcatagac attagtaatt caaatgggaa aaaaacgcaa gcgttgcatc tcacttcgcc ttgtttaaca
494 T L D A K T G I I D I S N S N G K K T Q A L H F T S P L L T
1801 gcaggaaactg aatcacgtga acgtttaact aatggtaaat actcttatat taataagtta aaattcggac gtgtaaaaaa ctggcaagtt
524 A G T E S R E R L T N G K Y S Y I N K L K F G R V K N W Q V
1891 acagatggag aggctagttc taaattagat ttctctaaag ttattcagcg tgtagccgag acagaaggca cagacgagat tggcttaata
554 T D G E A S S K L D F S K V I Q R V A E T E G T D E I G L I
1981 gtaaatgcaa aagctggcaa tgacgatac tttgttggtc aaggtaaaat gaattattgat ggtggagatg gacacgatcg tgtcttctat
584 V N A K A G N D D I F V G Q G K M N I D G G D G H D R V F Y
2071 agtaaaagac gaggatttgg taatattact gtagatggtg cgagtgcac agaagcaggc agttatacag ttaatcgtaa ggttgctcga
614 S K D G G F G N I T V D G T S A T E A G S Y T V N R K V A R
2161 ggtgatatct accatgaagt tgtgaagcgt caagaaacca aggtgggtgaa acgtactgaa actatccagt atcgtgatta tgaattaaga
644 G D I Y H E V V K R Q E T K V G K R T E T I Q Y R D Y E L R
2251 aaagttgggt atgggtatca gtctaccgat aatttgaat cagtagaaga agtaattggt tctcaattta atgatgtatt caaaggttct
674 K V G Y G Y Q S T D N L K S V E E V I G S Q F N D V F K G S
2341 aaattcaacg acatattcca tagtgggtgaa ggtgatgatt tactcgatgg tgggtgctgg gacgaccgct tgtttggtgg taaaggcaac
704 K F N D I F H S G E G D D L L D G G A G D D R L F G G K G N

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2431 gatcgacttt ctggagatga aggcgatgat ttactcgatg gcggttctgg tgatgatgta ttaaatggtg gtgctggtaa tgatgtctat
734 D R L S G D E G D D L L D G G S G D D V L N G G A G N D V Y

2521 atctttcggg aaggtgatgg taatgatact ttgtacgatg gcacgggcaa tgataaatta gcatttgcag atgcaaatat atctgatatt
764 I F R K G D G N D T L Y D G T G N D K L A F A D A N I S D I

2611 atgattgaac gtaccaaga gggattata gttaaacgaa atgatcattc aggtagtatt aacataccaa gatggtacat aacatcaaat
794 M I E R T K E G I I V K R N D H S G S I N I P R W Y I T S N

2701 ttacaaaatt atcaaagtaa taaaacagat cataaaattg agcaactaat tggtaaagat ggtagttata tcacttccga tcaaattgat
824 L Q N Y Q S N K T D H K I E Q L I G K D G S Y I T S D Q I D

2791 aaaattttgc aagataagaa agatgggtaca gtaattacat ctcaagaatt gaaaaagctt gctgatgaga ataagagcca aaaattatct
854 K I L Q D K K D G T V I T S Q E L K K L A D E N K S Q K L S

2881 gcttcggaca ttgcaagtag cttaaaataag ctagttaggt caatggcact atttgggtaca gcaaatagtg tgagttctaa cgccttacag
884 A S D I A S S L N K L V G S M A L F G T A N S V S S N A L Q

2971 ccaattacac aaccaactca aggaattttg gctccaagtg tttagtatt taatttacta gacaatatca ccacccatat cattgggtat
914 P I T Q P T Q G I L A P S V -

3061 agattatgaa actagtata tgggtggtga tacttcttta attagactta atttacaac ccttaatagt aatttagtta tgatagatta
M G G D T S L I R L N L Q T L N S N L V M I D

3151 tgctcaacaa cctgctctat ctgctctggt tatccttgcc aaatactatg gtatttctgc aagtcagca gacattatgc atcgactggc
Y A Q Q P A L S A L V I L A K Y Y G I S A S P A D I M H R L

3241 aaaaaagttg
A K K L

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Figure 11: Nucleotide and amino acid sequence of the A subunit of the RTX toxin from Dalton 2d / *M. bovis*.

Putative ribosome binding site shown in bold and underlined.

Putative start codon shown in bold.

Numbering for both the nucleotide and amino acid sequences are shown down the left hand side.

Upstream of the A subunit open reading frame is a portion of the coding region for the C subunit (nucleotide 1 to 195) and downstream of the A subunit is a small portion of the B subunit coding region (nucleotide 3080 to 3250).

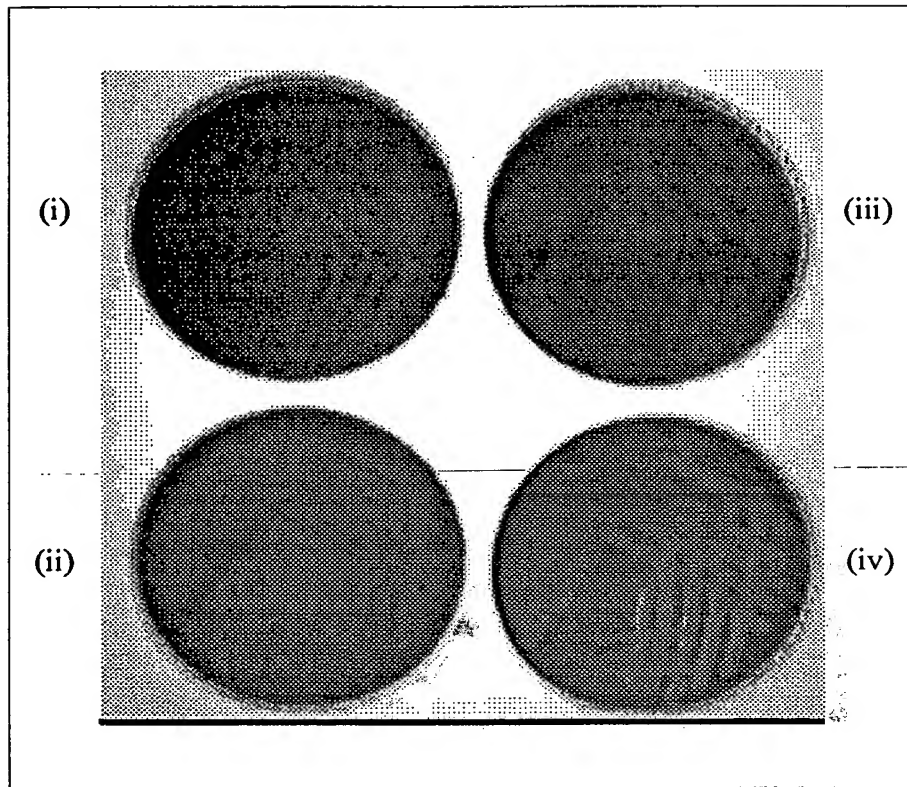


Figure 12: Complementation analysis of the RTX toxin operon of *E. coli* with the A subunit of the RTX toxin of *M. bovis*.

- Plates: (i) pLG900 in DH5 α
(ii) pLG900 / A subunit(-) in DH5 α
(iii) *M. bovis* A subunit / pGem-Teasy in DH5 α
(iv) pLG900 / A subunit(-) and *M. bovis* A subunit / pGem-Teasy in DH5 α

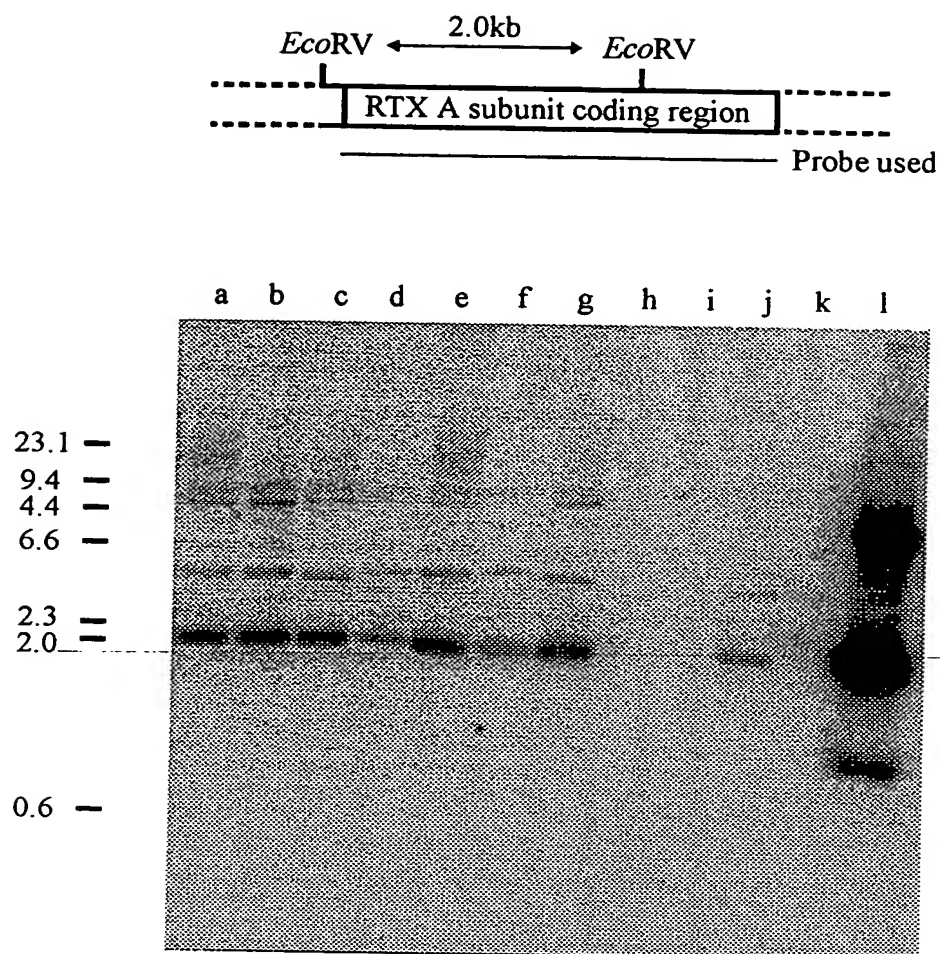


Figure 13: Southern hybridisation carried out to determine whether the RTX A subunit cloned from Dalton 2d was conserved across all seven serotypes of *M. bovis*. Shown above the southern blot is a diagram of the sites used in the genomic digests and the area to which the probe should bind.

- Lanes:
- a. S276R
 - b. 3WO7
 - c. Dalton 2d
 - d. R593L
 - e. Tat849
 - f. 218R
 - g. Fleur462
 - h. space
 - i. Gordon 26L3 (non-haemolytic strain of *M. bovis*)
 - j. UQV148NF (haemolytic strain of *M. bovis*)
 - k. positive control

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